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EDITORIAL

Growth factor receptors and related signalling pathways as targets for novel treatment strategies of hepatocellular cancer

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Abstract

Growth factors and their corresponding receptors are commonly overexpressed and/or dysregulated in many cancers including hepatocellular cancer (HCC). Clinical trials indicate that growth factor receptors and their related signalling pathways play important roles in HCC cancer etiology and progression, thus providing rational targets for innovative cancer therapies. A number of strategies including monoclonal antibodies, tyrosine kinase inhibitors ("small molecule inhibitors") and antisense oligonucleotides have already been evaluated for their potency to inhibit the activity and downstream signalling cascades of these receptors in HCC. First clinical trials have also shown that multi-kinase inhibition is an effective novel treatment strategy in HCC. In this respect sorafenib, an inhibitor of Raf-, VEGF- and PDGF-signalling, is the first multi-kinase inhibitor that has been approved by the FDA for the treatment of advanced HCC. Moreover, the serine-threonine kinase of mammalian target of rapamycin (mTOR) upon which the signalling of several growth factor receptors converge plays a central role in cancer cell proliferation. mTOR inhibition of HCC is currently also being studied in preclinical trials. As HCCs represent hypervascularized neoplasms, inhibition of tumour vessel formation *via* interfering with the VEGF/VEGFR system is another promising approach in HCC treatment. This review will summarize the current status of the various growth factor receptor-based treatment strategies and in view of the multitude of novel targeted approaches, the rationale for combination therapies for advanced HCC treatment will also be taken into account.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide. With an alerting 5 years relative survival rate of about 7% HCC is estimated to cause more than half a million deaths annually. HCC is the third most common cause of cancer deaths. The disease is most prevalent in Eastern and Southeastern Asia, and Middle Africa, with more than half of the patients being reported from China[1]. However, also in the developed countries the incidence of HCC dramatically increased in the past decades, mainly due to the increasing prevalence of chronic hepatitis C[2].

HCC is one of the few cancers with well-defined major risk factors. In Western countries > 80% of HCC develop in livers with cirrhosis mainly due to chronic hepatitis C, alcohol abuse, chronic hepatitis B or hemochromatosis. Especially in developed countries there is increasing concern regarding the epidemic of obesity which is associated with type 2 diabetes and other features of the metabolic syndrome and which frequently leads to non-alcoholic steatohepatitis (NASH). Here NASH may become to be one of the major causes of cirrhosis; diabetes and NASH are risk factors for developing HCC[2-4]. Rarer causes are cirrhosis due to hemochromatosis, autoimmune liver diseases or congenital disorders of metabolism. Cirrhosis in a setting of chronic liver cell injury, with inflammation, hepatocyte necrosis and regeneration, is a particular breeding ground for hepatocyte dedifferentiation and HCC[5]. In developing

countries, HCC frequently arises in non-cirrhotic livers, mostly on the basis of congenital infection with the hepatitis B virus which acts as mutagen due to insertion in the human genome and/or on the basis of aflatoxin exposure from contaminated food[2,4].

Unfortunately, the majority of patients suffer from advanced HCC at presentation. Therefore, curative treatment like local ablation, surgical resection or liver transplantation can be achieved in only a minority of HCC patients[6]. Local tumour destruction, chemoembolisation or systemic therapy are the treatment options of advanced HCC. Apart from transarterial chemoembolisation, which improves survival in well-selected patients with unresectable HCC, conventional palliative treatment options do not appear to improve overall outcome[5,6]. A recent meta-analysis of Simonetti and coworkers, who evaluated the results of randomized clinical trials of systemic and regional chemotherapy of HCC patients confirmed the disappointing results and revealed that nonsurgical therapies are more or less ineffective and do not prolong the survival of HCC patients, while further compromising quality of life[7].

Effective palliative treatment is hampered by the fact that advanced HCC represents a tumour entity which is extremely resistant to radiotherapy and conventional chemotherapy[8]. Moreover, the existing conventional chemotherapeutics are more or less non-selective cytotoxic drugs with significant systemic side-effects. Importantly, as most patients with advanced HCC have compromised liver function aggressive medical therapy regimens can not be applied. Thus, usually no effective therapy can be offered to these patients.

Because of the lack of any survival benefit of treatment with conventional drugs, new agents and novel therapeutic strategies are urgently needed to improve palliative treatment, prolong life expectancy and improve quality of life in patients with advanced HCC.

POTENTIAL TARGETS FOR FUTURE HCC THERAPIES

Growth factors and their related receptors are interesting targets for future therapeutic approaches. During foetal life, a large number of growth factors including the epidermal growth factor (EGF), insulin-like growth factors (IGFs), the hepatocyte growth factor (HGF), the vascular endothelial growth factor (VEGF), the fibroblast growth factor (FGF), the platelet-derived growth factor (PDGF) and the transforming growth factors -α and -β (TGF-α, TGF-β) are produced in the liver. In the adult normal liver many of them decline or are even absent. On the other hand adult hepatocytes are able to upregulate the production of particular growth factors like EGF, TGF-α, IGFs and VEGF, when liver regeneration is required after injury or damage[9,10]. This normally transient upregulation is dysregulated in the chronic injured liver leading to sustained mito-oncogenic signalling. Thus, dysregulation of the growth factor production and growth factor receptor signalling of adult hepatocytes plays an important role in hepatocarcinogenesis.

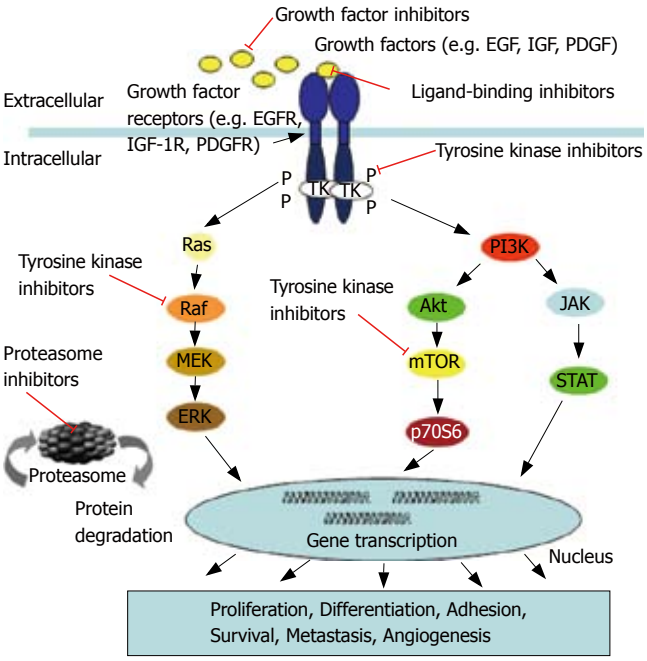


Figure 1 Major growth factor receptor signalling pathways in HCC.

Furthermore, members of the fibroblast growth factor and platelet-derived growth factor families, FGFs and PDGF play important roles in promoting liver fibrosis and HCC growth[11,12]. Like HGF, these growth factors are produced and released from non-hepatocyte sources like activated hepatic stellate cells, myofibroblasts, endothelial cells, Kupffer cells and bile duct epithelia and do also contribute to hepatocarcinogenesis.

GROWTH FACTOR RECEPTOR RELATED SIGNALLING PATHWAYS IN HCC CELLS

In the last decade some of the relevant pathways in cancer biology have been deciphered and there is emerging evidence that particularly growth factor receptors and their related downstream signalling pathways play a pivotal role in the development and maintenance of various cancers including HCC. Among the most critical cellular signalling pathways which support hepatocarcinogenesis are the receptor tyrosine kinase-activated pathways which include the rat sarcoma/rat sarcoma-activated factor (raf) /mitogen activated protein kinase/extracellular regulated kinase kinase/extracellular regulated kinase pathway (Ras/Raf/MEK/ERK), the Janus kinase/signal tranducers and activator of transcription pathway (JAK/STAT)s, and the phosphatidyl inositide 3 kinase/protein kinase B (AKT) /mammilian target of rapamycin pathway (PI3K/AKT/mTOR) (Figure 1).

Ras/Raf/MEK/ERK pathway

The Ras/Raf/MEK/ERK pathway appears to be one of the most significant cellular signalling sequences in the development and maintenance of hepatocellular cancer. This pathway transduces extracellular signals from ligand-bound tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR), the insulin-like growth

factor receptor (IGFR), the vascular endothelial growth factor receptor (VEGFR) or the platelet-derived growth factor receptor (PDGFR) to the nucleus in a series of specific phosphorylation events, starting with the activation of Ras which in turn activates serine threonine kinases of the Raf-family[1]. Activated Raf phosphorylates MEK 1/2 kinases which finally activate the extracellular regulated kinases ERK 1/2. Once activated, ERK 1/2 translocates to the nucleus where it acts as a regulator of gene expression of various proteins, including those for cell cycle progression, apoptosis resistance, extracellular matrix (ECM) remodeling, cellular motility angiogenesis and drug resistance[13]. Dysregulation of this crucial pathway occurs due to oncogenic transformation of Ras and Raf isoforms, or overexpression and/or overactivation of the Ras and Raf genes. In a recent study overexpression of the *Raf*-1 gene was shown in 50% of HCC biopsies, while increased activation of the Raf-1 protein was found even in 100% of the $n = 30$ evaluated HCC biopsies[14]. However, significant contribution of the proliferative Ras pathway to the development of HCC has long been a matter of debate. Previous studies suggested that activation of the Ras pathway might only be important in rodent, but not in human HCC, because of the low incidence of Ras gene mutations found in human HCC, while activation of the Ras cascade also occurred in the presence of wild-type Ras[15]. Recently, the components of the Ras cascade in human HCCs were characterized, demonstrating a downregulation or loss in the expression of specific members of the RAS inhibitor family. Among them the RAS association family 1 gene A (RASSF1A) and its homologue NORE1A in 100% of the $n = 35$ examined HCC[16]. The inactivation of these inhibitors resulted in a persistent activation of the Ras pathway and the authors suggested that the use of Ras inhibitors may thus be an interesting therapeutic modality for future treatment of HCC.

JAK/STAT pathway

The same holds true for the JAK/STAT pathway which plays an important role in cellular processes like differentiation, proliferation, and apoptosis[17]. STATs are latent in the cytoplasm and become activated through tyrosine phosphorylation which typically occurs through JAKs or growth factor receptor tyrosine kinases. Activated STATs enter the nucleus and serve as transcription factors. As to apoptosis and cell cycle related genes the transcriptional changes induced by STATs are similar to those described for ERK1/2.

In normal cells, ligand-dependent activation of STATs is transient, but in tumours the STAT proteins (in particular STAT-1, -3 and -5) are often constitutively activated[18,19]. This constitutive activation is partly due to inactivation of specific STAT inhibitors, the suppressors of cytokine signalling (SOCS), which normally balance and terminate STAT activity[20,21]. Thus, loss of activation of the STAT inhibitors such as cytokine-inducible SH2-protein (CIS), SOCS1, SOCS2, SOCS3, and SH2-containing phosphatases (SHP1) was shown to account for the constitutive activation of antiapoptotic and mitogenic STAT-3 and -5 in HCC[16].

In terms of both the Ras and the JAK/STAT pathway it may not be the increase in gene mutations of the respective pathway proteins, but the state of activation of these pathways due to an imbalanced interplay of activators and inhibitors, which accounts for the pivotal role of these pathways in HCC.

PI3K/AKT/mTOR pathway

The activated PI3K/AKT/mTOR pathway has only recently emerged as a novel contributor to (HCC) tumour development. PI3K associates with the intracellular domain of several growth factor receptors. Upon activation PI3K triggers the generation of phosphatidylinositol 3,4,5-triphosphate (PIP_3) which provokes the subsequent activation of AKT, a serine/threonine kinase which regulates multiple cellular target proteins. Among these proteins is proapoptotic BAD, which becomes inactivated by phosphorylation, and also the mammalian target of rapamycin (mTOR) subfamily of proteins, which become activated by AKT. mTOR proteins regulate the phosphorylation of p70 S6 serine-threonine kinase and the translational repressor protein PHAS-1/4E-BP. Both proteins regulate the translation of proliferation- and angiogenesis-relevant proteins, such as c-myc, cyclin-D1, ornithine decarboxylase, hypoxia-induced factor 1-α, and are indirectly involved in the expression of VEGF[1,22].

In nontransformed cells the PI3K/AKT/mTOR pathway is controlled by the phosphatase and tensin homolog deleted on chromosome ten (PTEN), a tumour suppressor which inhibits this pathway by reversing the PI3K reaction and blocking AKT activation. Mutation or silencing of the PTEN gene leads to activation of the pathway and promotes carcinogenesis. PTEN expression is reduced or absent in almost half of the studied HCCs, and hepatocyte-specific abrogation of PTEN expression in mice results in the development of HCC[23]. Thus, constitutive activation of this pathway can be due to enhanced stimulation of growth factor receptors, like EGFR and IGFRs, but can also result from decreased PTEN expression[1]. In non-HCC tumour models loss of PTEN expression has been demonstrated to negatively influence the sensitivity towards EGFR-TK inhibition by gefitinib[24]. Thus, it will be interesting to evaluate if this also holds true for HCC. If so, PTEN expression can serve as a novel marker for predicting the response to tyrosine kinase inhibition-based treatment strategies in HCC and therapeutic strategies in which normal PTEN expression can be restored, should be an attractive approach for combined therapeutic strategies in HCC treatment in the future.

Moreover, recent work by Boyault and coworkers demonstrated that in specific subgroups of HCC patients, namely those that are infected with low copy number hepatitis B virus (HBV) and concomitant overexpression of genes expressed in fetal liver, as well as those infected with a high copy number of HBV, but concomitant mutations in the catalytic domain of the phosphoinositide-3 kinase (*PIK3CA*) and the tumor suppressor protein 53 (*TP53*), there is a frequent upregulation of AKT expression and activation, rendering

Table 1 Agents for anti-EGFR-based therapy of solid tumours

Name	Target	Mechanism	Current status
Small molecule inhibitors			
Gefitinib (Iressa)	EGFR	Reversibly acting tyrosine kinase inhibitor	Approved for NSCLC with restricted indications Phase I for HCC[133]
Erlotinib (Tarceva)	EGFR	Reversibly acting tyrosine kinase inhibitor	Approved for NSCLC and pancreatic cancer Phase Ⅱ for hepatocellular cancer[33]
EKB-569	EGFR	Irreversibly acting tyrosine kinase inhibitor	Phase Ⅰ/Ⅱ for colorectal cancer[134] Phase Ⅱ for NSCLC[135]
Lapatinib (Tykerb)	EGFR, erbB2	Reversibly acting tyrosine kinase inhibitor	Phase Ⅲ for breast cancer Phase Ⅱ for HCC[136]
Canertinib (CI-1033)	Pan-erbB	Irreversibly acting tyrosine kinase inhibitor	Phase Ⅱ for SCC and ovarian cancer[137]
BMS-599626	EGFR, erbB2	Reversibly acting tyrosine kinase inhibitor	Phase Ⅱ for HCC [138]
Monoclonal antibodies			
Cetuximab	EGFR		Approved for colorectal cancer Phase Ⅲ for head and neck cancer, NSCLC and pancreatic cancer Phase Ⅱ for HCC[139]
Trastuzumab	erbB2		Approved for breast cancer
ABX-EGF	EGFR		Phase Ⅲ for colorectal-, head and neck-, and renal cell cancer
Matuzumab (EMD 72000)	EGFR		Phase I/Ⅱ for NSCLC[140], ovarian-[141], pancreatic cancer[142]

these patients especially susceptible to therapeutic approaches that inhibit the AKT-pathway[25].

Due to the importance of the above-described signalling pathways linked to growth factor receptors in the development and maintenance of (hepatocellular) cancer, several attempts have been undertaken to develop specific inhibitors which either block the communication of growth factors and their cognate receptors using antibodies or growth factor trapping decoy receptors, or by interrupting the transmission of growth factor receptor which signals to the respective downstream signalling cascades by membrane permeable small molecule inhibitors which block the intrinsic receptor tyrosine kinase activity. The following section will provide a concise overview of selected agents which are currently in the development and testing for the targeted treatment of solid tumours and HCC.

EGFR-based strategies

The crucial role of epidermal growth factor receptor (EGFR) in tumour proliferation and its overexpression in several solid tumours have provided the rationale for targeting and interrupting this key signalling network. EGFR blockade through monoclonal antibodies and tyrosine kinase inhibitors has translated into promising evidence of clinical benefit in gastrointestinal tumours, particularly colorectal cancer[26]. EGFR is expressed in a high proportion of HCCs, and EGFR-inhibitors, such as the monoclonal antibody cetuximab or the tyrosine kinase inhibitors gefitinib, erlotinib or ANAPD have been shown to inhibit HCC growth and metastasis formation *in vitro*[27-31] and *in vivo*[32]. Recently, Philips and coworkers conducted a phase Ⅱ trial with erlotinib for advanced HCC and could demonstrate very encouraging results, as they observed good response rates in approx. One third of the treated patients and a prolonged survival with mild and tolerable side effects after treatment with a dose of 150 mg/d[33].

Despite the encouraging findings on the general suitability of anti-EGFR-based-approaches for the treatment of HCC, only few clinical trials have been conducted so far. Most of our current knowledge on the clinical benefit of anti-EGFR-based therapies originates from studies on other tumour entities, such as colorectal cancer, renal cell carcinoma and non-small cell lung cancer (NSCLC). Nevertheless, at present several clinical trials evaluate the efficacy of anti-EGFR-interventions for the treatment for HCC (www.clinical-trials.gov), and based on the data that are available so far there is hope that anti-EGFR mono- or combination therapies will qualify for improving the treatment of advanced HCC in the near future. Currently, another phase Ⅱ trial is conducted in patients with advanced HCC which evaluates the efficacy of a combination of erlotinib and the anti-angiogenic VEGF-blocking antibody bevacizumab (NIH, NCT00365391). Dual-targeting of the HCC cells and their nutrient supply *via* the surrounding vasculature may improve the antitumoural effects as compared to monotherapy with either erlotinib or bevacizumab alone[34].

Thus the majority of the currently tested anti-EGFR-based approaches are increasingly combined either with conventional cytostatics or with other targeted-agents[28,29,35,36]. The rationale for applying combination therapies is the existence of multilevel receptor cross-stimulation or of redundant signalling pathways that lead to neoplasia. Blocking only one of these pathways allows others to act as salvage or escape mechanisms for cancer cells. Preclinical evidence of synergistic antitumour activity achievable by combining targeted agents that block multiple signalling pathways has recently emerged[37-41]. The multi-target approach can be accomplished by using either combinations of selective agents or agents which interfere with various targets[42]. Table 1 shows the current status of anti-EGFR-strategies for the treatment of solid tumours including HCC.

IGF/IGFR-based strategies

There is compelling evidence that both insulin-like growth factors IGF-Ⅰ and -Ⅱ and their receptor tyrosine kinase, IGF-1R, are involved in the development and

Table 2 Agents for anti-IGF-1R-based cancer treatment

Name	Target	Mechanism	Current status
Small moelcule inhibitors			
INSM-18	IGF-1R and HER2	Substrate competitive inhibitor	Phase Ⅰ[44]
NVP-AEW541	IGF-1R	ATP-competitive inhibitor	Preclinical[63]
NVP-ADW742	IGF-1R	ATP-competitive inhibitor, activation of proapoptotic pathways	Preclinical[62]
BMS-536924	IGF1R and IR	ATP competitive inhibitor,	Preclinical[143]
Cyclolignans	IGFR-1R	IGF competitive inhibitor	Preclinical[144]
Antibodies			
CP-751, 871	IGF-1R	IGF1R downregulation	Phase I for multiple myeloma Phase Ⅱ for Breast-[145], lung-[146], and prostate[147] cancer
A12	IGF-1R	IGF1R down-regulation, apoptosis, cell cycle arrest	Phase Ⅰ[148]
scFv-Fc	IGF-1R	IGF1R downregulation	Preclinical[149]
AVE-1642	IGF-1R	IGF1R downregulation, cell-cycle arrest, induction of apoptosis	Preclinical[69]

progression of cancer[43-46]. Interaction of IGF-Ⅰ and -Ⅱ with the IGF-1R plays a pivotal role in tumourigenesis, proliferation and spread of many cancers, by promoting cell cycle progression, preventing apoptosis, and by regulating and maintaining the tumourigenic phenotype. A wide variety of tumours including HCC show abnormal, or enhanced expression of IGFs and IGF-1R, which has been correlated with disease stage, reduced survival, development of metastases and tumour de-differentiation[47-49]. In men, obesity and diabetes are clearly associated with an increased risk of HCC, and this seems to be due to alterations in the metabolism of endogenous hormones, including sex steroids, insulin and the IGF/IGFR system. Thus, a promising approach of innovative HCC treatment may be the blockade of the IGF/IGFR, but also the mTOR-signalling system, which is functionally upregulated in HCC cells *in vitro*[50-52] and *in vivo*[47], and which has been shown to exert strong stimulatory effects on the growth of hepatoma cells[48]. In addition to the increased expression of IGF-1R and IGFs, a simultaneous reduction of IGF binding protein expression (IGFBP) and enhanced proteolytic cleavage of IGFBPs often occurs. Both mechanisms lead to an excessive increase in the amount of bioactive IGF[50,53] which further enhances the mito-oncogenic effects of IGFR-signalling in HCC and other cancer cells. The expression of IGF-1R is very low in normal hepatocytes that are poorly responsive to IGFs, whereas significant expression is found in Kupffer, endothelial and hepatic stellate cells[50].

Several approaches have demonstrated the therapeutic potential of interfering with IGF-1R mediated signalling *in vitro* and *in vivo*, including the use of IGF-1R blocking antibodies, IGF-1R antisense oligonucleotides or IGF-1R siRNA[54-57].

Recently, we and others introduced the potent and selective IGF-1R tyrosine kinase inhibitor, NVP-AEW541, as promising novel agent for the therapy of several cancers[58-60], including HCC[61]. The antineoplastic properties of NVP-AEW541 and related compounds such as NVP-ADW742[62] have been demonstrated in preclinical studies on Ewing´s sarcoma-bearing mice[63], fibrosarcoma, breast cancer and musculoskeletal carcinoma[64-66]. Specific IGFR-antibodies have also shown to suppress prostate and breast cancer cell growth in a recent preclinical study[67]. The clinically most advanced anti-IGFR antibody is CP-751,871 which is currently being tested in three phase Ⅱ trials for advanced breast cancer, NSCLC and prostate cancer (www.clinical-trials.gov). Importantly, IGFR-inhibition appears to be well-tolerated in the preliminary clinical studies conducted so far[63,68,69]. Safety is important, since IGFR-based inhibition has long been regarded as a high risk intervention, because of the high homology of the IGF-1R receptor with the related insulin-receptor, and the fear that especially IGF-1R-TK inhibitors might do also block the insulin receptor which could lead to insulin resistance and overt diabetes[70]. However, the current *in vivo* studies did not confirm this apprehension, resulting in growing interest in anti-IGFR-based therapies[71].

It is widely accepted that a therapy which inhibits IGF signalling may have to be combined with other therapies to enhance the antiproliferative overall-effect, since crosstalk between the signalling of IGF and other growth factor receptors have already been shown to be able to attenuate the antineoplastic effects of a respective monotherapeutic approach[72]. Thus, we and others could show that IGFR and concomitant EGFR-inhibition or conventional chemotherapy enhances the antineoplastic effect of the respective monotherapies[28,29,61]. Especially, dual-targeting EGFR and IGF-1R is a promising approach for future treatment of HCC. The rationale for this particular combination is derived from observations that in HCC cells the EGFR-system is activated by the IGF/IGFR-system *via* receptor cross-talk leading to mito-oncogenic EGFR-tyrosine kinase activity[73,74]. Thus inhibition of IGF-2-related signalling leads to sensitization of HCC cells to anti-EGFR-treatment with gefitinib[72], and it was postulated that inhibition of IGF/IGF-1R-signalling may not only enhance the effects of gefitinib treatment, but may also help to overcome resistance to anti-EGFR-based therapy of HCC[75]. Table 2 summarizes the most promising IGF/IGFR-targeted agents which are currently under intense investigation in preclinical and early clinical trials.

VEGF/VEGFR-based strategies

VEGF is the key angiogenic factor in tumours. The VEGF gene and protein have been reported to be transcribed, expressed, and secreted by HCC cells[76]. Endothelial

Table 3 Agents for anti-VEGF/VEGFR-based therapy of solid tumours

Name	Target	Mechanism	Current status
Small molecule inhibitors			
Sunitinib (Sutent)	PDGFR, VEGFR, c-KIT, FLT-3	Tyrosine kinase inhibitor	Approved for advanced renal cell carcinoma and GIST (with restricted indications)[87,150] Phase I for HCC[151]
Zactima (ZD6474)	VEGFR, EGFR	Tyrosine kinase inhibitor	Phase Ⅲ for NSCLC[156] Phase Ⅱ for thyroid cancer[157]
Vatalanib (PTK787/ZK 222584)	VEGFR, PDGFR, C-KIT	Tyrosine kinase inhibitor	Phase Ⅱ/Ⅲ for colorectal cancer[152] Phase I for HCC[153]
Anti-ligand targeting			
Bevacizumab	VEGF	VEGF-neutralizing antibody	Approved for colorectal cancer Phase Ⅲ for NSCLC[166] Phase Ⅱ for HCC[154]
VEGF trap	VEGF	Soluble decoy receptor which neutralizes all VEGF A isoforms	Phase I for advanced solid tumours[155]

cells which line tumour vessels express VEGFR-1 and VEGFR-2 which communicate to stimulate each other in a feedback-loop[77]. Given that VEGF protein expression is related to HCC grade[78] and given that the degree of microvascular density correlates with HCC grade[79,80], it is comprehensible that inhibitors of VEGF signalling are promising therapeutic agents for HCC treatment.

Bevacizumab is a humanized murine monoclonal anti-VEGF antibody which has entered the clinic for treatment of cancer. Standard cytostatic treatment plus bevacizumab significantly increased survival in metastatic colorectal cancer compared to standard treatment alone in a phase Ⅲ clinical trial[81] which led to its approval for treatment of colorectal cancer 2005. Comparable results were obtained in a recent phase Ⅲ clinical with bevacizumab for treatment of NSCLC. This study had to be interrupted because of the obvious survival advantage of patients in the bevacizumab arm[82]. Bevacizumab monotherapy is currently being tested in patients with unresectable HCC[83] (National Cancer Institute: NCT00162669). Moreover, a phase Ⅱ trial is currently being conducted testing the efficacy of bevacuzimab in combination with capcetabine and oxaliplatin in patients with advanced HCC. An intermediate evaluation of this trial is encouraging and shows that this combination is tolerable to patients with advanced HCC and cirrhosis[84]. As described earlier, bevacizumab is also currently tested in combination with the EGFR-tyrosine kinase inhibitor erlotinib (see above).

In addition, several agents that inhibit the tyrosine kinase activity of VEGFR have been synthesized by combinatorial chemistry. Recent clinical studies revealed the suppression of HCC growth by vatalanib (PTK787/ZK 222584), which inhibits the activities of VEGFR-1 and -2 and has shown antineoplastic effects in other solid tumours[85,86]. Another interesting agent is the tyrosine kinase inhibitor sunitinib, which inhibits the VEGFR- as well as the PDGF-βR, c-KIT and FLT-3 tyrosine kinases. Sunitinib has been approved for the treatment of renal cell carcinoma[87]. With restricted indications sunitinib is also approved for the therapy of gastrointestinal stromal tumours (GIST)[88] and is currently tested in phase I and Ⅱ trials for HCC (NIH: NCT00361309; NCT00247676).

Another promising approach is the use of dual-targeting tyrosine kinase inhibitors, which inhibit less related tyrosine kinases, such as NVP-AEE788 or zactima (ZD6474) which target both the VEGFRs and the EGFR. In recent *in vivo* studies of non-HCC tumour models (colon, cholangiocarcinoma, prostate, NSCLC) NVP-AEE788 displayed significant antineoplastic efficacy. These agents can inhibit both tumour cell proliferation and survival by blocking hepatoma EGFR and angiogenesis by inhibiting endothelial VEGFR. These promising recent results warrant further evaluation in clinical trials[89-92]. For zactima successful testing in clinical trials has already been reported for non-HCC tumour entities like NSCLC and thyroid cancer[93,94]. Table 3 summarizes the current status of anti-VEGF/VEGFR-based approaches in the treatment of solid tumours including HCC.

OTHER MULTI-KINASE AND GROWTH FACTOR RECEPTOR INDEPENDENT INHIBITORS

Multi-kinase inhibition

The novel bi-aryl urea sorafenib is an orally available multi-kinase inhibitor which targets kinases of wild-type B-Raf, mutantV559EB-Raf and C-Raf, thus blocking tumour growth. Furthermore, sorafenib potently inhibits receptor tyrosine kinases involved in angiogenesis, including human vascular endothelial growth factor receptors-2 and -3 (VEGFR-2/-3) and PDGF-βR. The principal mechanism of action of sorafenib is the competitive inhibition of ATP-binding to the catalytic domains of the respective kinases[95]. However, the fact that sorafenib is an oral multi-kinase inhibitor, with effects on several molecular targets in addition to the Raf isoforms makes it difficult to determine which of these targets contributes most to its anti-tumour activity in particular tumour types.

A recent phase Ⅱ HCC clinical trial, which identified an association between high baseline tumour p-ERK levels and improved response to sorafenib, suggests that inhibition of the Raf/MEK/ERK pathway is central to sorafenib's mode of anti-tumour action in HCC[96]. If this generally holds true for HCC remains to be determined. In other tumour entities the antineoplastic potency of

sorafenib appears to be mainly due to its antiangiogenic activity[97,98].

It is of particular clinical importance to have reliable markers to individually predict the treatment outcome. It has been suggested that rash, which is commonly associated with EGF-pathway inhibition, could be predictive of treatment outcome, and that the onset of rash could be used for optimal dose titration[99]. This might also be effective in treatment with sorafenib, as it is an inhibitor of Raf kinase, which is a downstream effector molecule of the EGFR signalling pathway. A recent report combining data from four phase I trials supported this hypothesis. Patients receiving sorafenib dosed at or close to the recommended dose of 400 mg bid, and experiencing skin toxicity and/or diarrhea, had a significantly increased time to progression compared with patients without such toxicity[100].

Sorafenib inhibits the proliferation of a variety of human cancer cell lines and retards tumour growth in related xenograft models of NSCLC, breast, colon and pancreas carcinoma[13,101,102]. Sorafenib is also active in otherwise fairly therapy resistant cholangiocarcinoma cells. Here, it over-additively enhanced the antineoplastic effects of cytostatics such as doxorubicin or the histone deacetylase inhibitor MS-275 and acts synergistically with IGFR blockade[103,104]. Recent *in vitro* studies by our group confirmed the synergistic antiproliferative effects of a combination treatment with sorafenib and MS-275 in hepatocellular carcinoma models. Proliferation studies, with either Hep-G2 or Huh-7 cells, resulted in half-maximal growth inhibition at a sorafenib concentration of 1.6 ± 0.3 μmol/L (Hep-G2) and 4.4 ± 0.2 μmol/L (Huh-7), respectively. The IC_{50} of MS-275 amounted to 1.2 ± 0.1 μmol/L in Hep-G2 cells and 0.9 ± 0.2 μmol/L in Huh-7 cells. Co-application of sub-IC_{50} concentrations of sorafenib and MS-275 for three days resulted in significant over-additive growth inhibition of Huh-7 cells, while in Hep-G2 cells a rather additive growth inhibitory effect was observed (Figure 2). Our data support the idea of dual-targeting hepatocellular carcinoma cells for enhanced treatment efficacy and show that multi-kinase inhibition plus histone deacetylase inhibition appear to be a promising combination, warranting further elucidation in clinical trials.

A series of clinical studies have tested sorafenib's antineoplastic potency in cancer patients. Phase I trials showed a favourable safety profile of 400 mg sorafenib administered twice daily for 12 wk in patients with advanced solid tumours (e.g. colon, ovary, breast, pancreas, kidney)[100,105]. Promising antitumour activities of sorafenib were observed in a phase Ⅱ clinical study of patients with advanced melanoma[106]. Most encouraging results were seen in phase Ⅱ and Ⅲ trails of patients with metastatic renal cell carcinoma (RCC) which led to approval in the US for advanced RCC[107].

Sorafenib has also been tested for the treatment of advanced HCC in phase Ⅱ and Ⅲ trials. In a phase Ⅱ trial on 137 patients with inoperable HCC the continuous oral application of sorafenib 400 mg bid in 4-wk cycles revealed a significant attenuation of HCC growth in 1/3 of the patients[108] resulting in a further evaluation

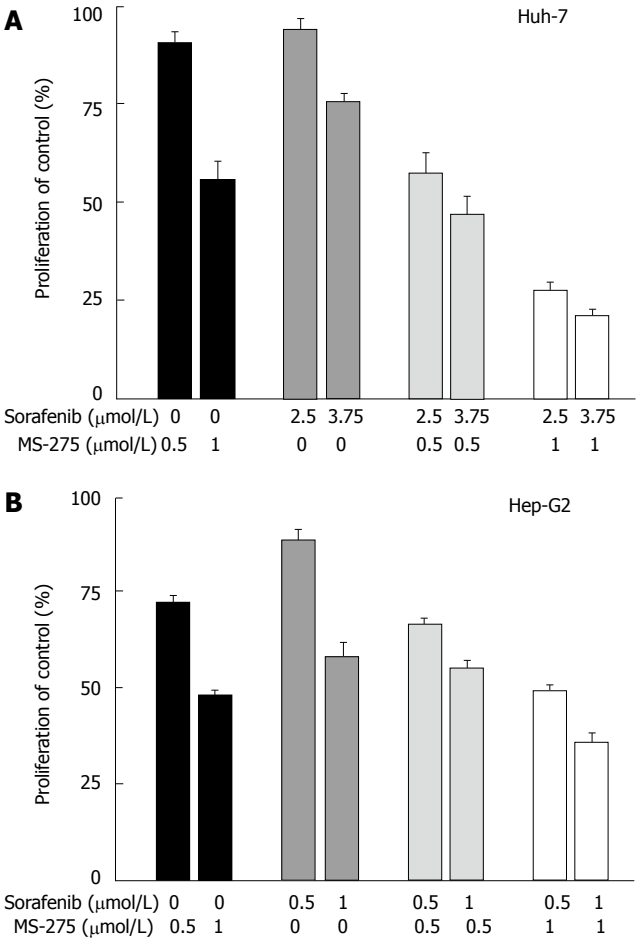


Figure 2 Antiproliferative effects of sorafenib-based combination treatment. **A**: Huh-7 and **B**: HEP-G2 cells were treated for 72 h with sub-IC_{50} concentrations of sorafenib and the histone deactylase inhibitor MS-275. Combination of both agents resulted in synergistic growth inhibition of Huh-7 cells, while rather additive growth inhibitory effects were observed in HepP-G2 cells (mean ± SEM).

in a randomized double-blinded phase Ⅲ trial with 602 patients with advanced HCC. An interim evaluation of this international multi-center SHARP-study (Sorafenib HCC Assessment Randomized Protocol) led to discontinuation, as the HCC patients treated with sorafenib achieved a significant survival benefit over the placebo-treated controls. Llovet *et al* presented the respective data for the Sharp investigators study group at the ASCO meeting in 2007 and showed that the treatment of advanced HCC patients with sorafenib leads to a 44% improvement in the overall survival as compared to the control group. The median overall survival in the sorafenib treated arm was 10.7 mo *vs* 7.9 mo in the control arm. Moreover, the median time to progression was almost doubled (5.5 mo in the sorafenib arm *vs* 2.8 mo in the control arm). The authors concluded that the effects of sorafenib treatment are clinically meaningful and establish sorafenib as first-line treatment for patients with advanced HCC[109]. Based on these findings, sorafenib has recently gained accelerated approval by the FDA for the treatment of advanced unresectable HCC.

mTOR inhibition

The natural antibiotic rapamycin is a potent inhibitor of mTOR[110]. Recently, three analogues of rapamycin with

superior pharmacokinetic and biological properties have been synthesized and tested in clinical trials for different malignancies. The cell cycle inhibitor-779 (CCI-779, temsirolimus) is a soluble ester analogue. RAD001 (40-O-[2-hydroxyethyl]-rapamycin, everolimus) is an orally bioavailable derivative of rapamycin, and finally AP23573, which is a non-pro-drug analogue of rapamycin. These agents have been tested successfully in early clinical trials for their antineoplastic potency and/or tolerability in various malignancies, such as renal, breast and lung cancers (CCI-779), or are currently being studied in open clinical trials for the treatment of colorectal, endometrial cancer, recurrent or refractory solid tumours, and brain tumours (RAD001, everolimus)[111-113]. AP23573 has been successfully tested in a phase Ⅱ trial in sarcomas[114] and two phase I studies in patients with refractory or advanced solid tumours, showing partial responses and disease stabilisation in individual patients[115].

In vitro as well as preclinical *in vivo* data of HCC show, that mTOR inhibition by rapamycin and analogues significantly reduces HCC growth and improves survival primarily *via* antiangiogenic effects[116]. A Phase Ⅰ/Ⅱ trial evaluating everolimus for advanced HCC is currently starting to recruit patients[117] (NIH, NCT00390195). Moreover, the use of rapamycin and analogues for combination treatment together with conventional cytostatic drugs such as doxorubicin or vinblastine has been demonstrated to additively or even synergistically enhance the antineoplastic potency of the respective monotherapeutic HCC treatment with either doxorubicin or vinblastine alone[118-120].

Taken together, the *in vitro* and preclinical *in vivo* data as well as the clinical trials conducted so far show that mTOR inhibitors, including the rapamycin analogues CCI-779, RAD001 and AP23573, are promising combination agents for future cancer therapy. They are well tolerated and can produce stable disease or even substantial responses in relapsed or conventional therapy resistant solid tumours[115].

Proteasome inhibition

Another interesting therapeutic approach for innovative cancer treatment is the inhibition of the 26S proteasome, which is a large protease that is present in both the nucelus and the cytoplasm of eukaryotic cells and functions as an identifier and destructor of proteins branded for destruction by the ubiquitin system. The so called ubiquitin-proteasome pathway (UPP) is the major non-lysosomal proteolytic system in eukaryotic cells and triggers degradation of proteins involved in cell cycle progression, apoptosis, nuclear factor kappaB (NF-κB) activation, and angiogenesis. UPP also degrades mutant, damaged, and misfolded proteins[121]. Since these signalling pathways are critical for cell survival and proliferation, especially in cancer cells, the inhibition of the proteasome has emerged as an attractive target for cancer therapy.

Bortezomib (Velcade™) is a proteasome inhibitor, which blocks multi-ubiquitinated protein degradation by reversibly and competitively inhibiting the active site threonine residue of the 26S proteasome[122]. Antineoplastic activity of bortezomib has already been shown in several *in vitro* and *in vivo* studies[104,123,124]. Bortezomib is the first proteasome inhibitor which has been approved for cancer therapy and is in use for the treatment of advanced multiple myeloma[121]. Based on the results of a phase Ⅱ trial on bortzezomib in the treatment of mantle cell lymphoma (ML) the FDA recently granted approval to bortezomib for the treatment of patients with ML (www.cancer.gov)[125]. Other cancers, including neuroendocrine tumours, RCC, NSCLC, or metastatic sarcomas have also been evaluated in recent phase Ⅱ clinical trials. In some of these studies a significant antineoplastic effect of monotherapy with bortezomib was observed, while in some other studies no or only marginal responses to single treatment with bortezomib were found[126-128]. However, in these cases it was recommended to investigate the role of bortezomib in combination with other antitumoural drugs. The rationale for using bortezomib in combination treatment regimes is that bortezomib's mode of action is mainly based on the inhibition of the NF-κB pathway, which has been shown to exert chemosensitizing effects when administered together with other antitumoural drugs. Combination treatment studies with encouraging results have been reported for lung cancer and lymphoma[129-131]. A phase Ⅰ/Ⅱ trial of bortezomib in patients with unresectable HCC was recently reported to result in disease stabilization in some patients and the treatment was generally well tolerated. In this study it was also suggested to especially focus on combination treatment strategies using bortezomib together with HCC-relevant cytostatics such as doxorubicin[132]. We have recently conducted an *in vitro* evaluation of bortezomib-based treatment of HCC cells. Our findings underline the suitability of bortezomib for the treatment of HCC-alone or in combination with sorafenib. In Huh-7 and Hep-G2 cells nanomolar concentrations of bortezomib induced a marked growth inhibition after three days of treatment. Moreover, the combination of bortezomib and sub-IC_{50} concentrations of sorafenib resulted in additive growth inhibition in both hepatocellular Huh-7 and hepatoma Hep-G2 cells (Figure 3). Thus, our data support the idea of dual-targeting hepatocellular carcinoma cells for enhanced treatment efficacy using bortezomib as a combinatory drug. Our data strengthen the conception of multi-kinase inhibition plus bortezomib to be a promising combination for future HCC treatment, warranting further elucidation in clinical trials. Table 4 summarizes the current status of multi-kinase inhibitors and growth factor independent inhibitors for the treatment of solid tumours.

CONCLUSION

The concept of targeted-therapies which specifically inhibit growth factor receptors and their related signalling pathways emerged to be a promising approach for the innovative and effective medical treatment of various cancers, including hepatocellular carcinoma. Thus, advanced HCC is no longer a tumour disease without specific medical treatment options. The recent findings and clinical trials clearly demonstrate that especially combination treatments inhibiting more than just one

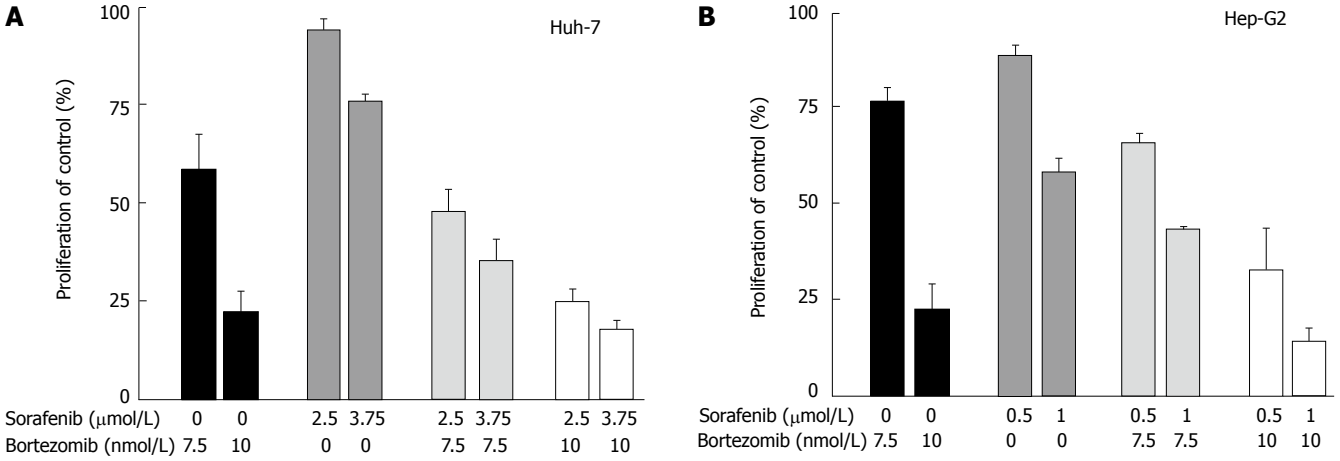


Figure 3 Antiproliferative effects of combination treatment with bortezomib and sorafenib in hepatocelluar carcinoma cells. **A**: Huh-7 and **B**: Hep-G2 cells were treated for 72 h with sub-IC_{50} concentrations of sorafenib and the proteasome inhibitor bortezomib. Combination of both agents led to additive growth inhibition both in Huh-7 as well as in Hep-G2 cells (mean ± SEM).

Table 4 Agents of multi-kinase- and growth factor independent inhibition for the therapy of solid tumours

Name	Target	Mechanism	Current status
Sorafenib	c-Raf-1, B-Raf, VEGFR, PDGFR	Tyrosine kinase inhibitor	Approved for advanced RCC Phase Ⅲ for advanced HCC[108] Phase Ⅱ for melanoma[106], breast cancer[158] and NSCLC[159] Phase Ⅰ for advanced solid tumours[160]
Everolimus (RAD001)	mTOR	Tyrosine kinase inhibitor	Phase Ⅱ for colorectal cancer[165] Phase Ⅰ / Ⅱ for advanced HCC[117,116] Phase Ⅰ for endometrial and brain tumours[111,112]
Temsirolimus (CCI-779)	mTOR	Tyrosine kinase inhibitor	Phase Ⅱ for metastatic breast cancer[161], advanced RCC[162], and mantle cell lymphoma[163] Phase Ⅰ for advanced solid tumours (e.g. colorectal, ovarian, lung cancer)[164]
AP23573	mTOR	Tyrosine kinase inhibitor	Phase Ⅱ for sarcomas of soft tissue and bone[114] Phase Ⅰ for advanced solid tumours
Bortezomib (Velcade)	Proetasome	Proteasome inhibitor	Approved for multiple myeloma and mantle cell lymphoma[125] Phase Ⅱ for colorectal cancer[166], neuroendocrine tumours[128], sarcoma[126], RCC[127], and NSCLC[129] Phase Ⅰ / Ⅱ for unresectable HCC[132] Phase Ⅰ for advanced solid tumours[167]

signalling pathway will be particularly efficient, as it leaves less mechanisms of escape for the tumour cells.

In addition, there are several other promising new drugs which are currently being tested or which should be investigated in future HCC trials. In this respect combinations with drugs such as multi-kinase inhibitors are particularly intriguing. Thus in the future agents like the multi-kinase inhibitor sorafenib will likely be combined with growth factor receptor inhibitors, proteasome inhibitors, HDAC inhibitors or cytostatics as to effectively control advanced HCC. The advantage of such novel combination therapies is their higher efficacy at lowered toxicity as compared to monotherapeutic approaches. The novel combination treatments will offer new chances for drug therapy even in HCC patients with underlying cirrhosis. Fortunately, most of the new drugs can be taken orally.

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REVIEW

Historical perspective of living donor liver transplantation

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Abstract

Living donor liver transplantation (LDLT) has gone through its formative years and established as a legitimate treatment when a deceased donor liver graft is not timely or simply not available at all. Nevertheless, LDLT is characterized by its technical complexity and ethical controversy. These are the consequences of a single organ having to serve two subjects, the donor and the recipient, instantaneously. The transplant community has a common ground on assuring donor safety while achieving predictable recipient success. With this background, a reflection of the development of LDLT may be appropriate to direct future research and patient-care efforts on this life-saving treatment alternative.

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INTRODUCTION

Living donor liver transplantation (LDLT) has been rapidly growing and evolving since its debut in 1989[1], while deceased donor liver transplantation (DDLT) had already been a standard procedure for a decade[2,3]. LDLT being the legitimate remedy for the refractory shortage of deceased donor liver grafts is characterized by its technical complexity and ethical controversies.

In 1963, Starzl described in detail three cases of DDLT. The first recipient was a 3-year-old boy with biliary atresia and died from intra-operative hemorrhage. The other two recipients were adult males with primary liver cancer. Both succumbed to pulmonary embolism 7 and 22 d after transplantation. These three cases, though ended up in hospital mortality, established the technical feasibility of liver transplantation in human[4]. Only four years later, long survivals were achieved in four DDLT recipients[5]. To become a reliable treatment modality for end-stage liver diseases of a number of etiologies, DDLT has taken two important steps: the clinical use of calcineurin inhibitor - cyclosporine A[6] and improvement of graft preservation techniques by hypothermic perfusion utilizing University of Wisconsin solution[7].

Soon after DDLT had become a standard clinical practice, it outstripped the supply of deceased donor liver grafts. The shortage of pediatric deceased donor liver grafts was even more marked. To overcome size disparity of the graft and the child recipient, reduced-size liver transplantation was devised by Bismuth in 1984[8]. Through extension of this concept, split-graft liver transplantation was then developed by Pichlmayr in 1988[9]. This enables transplanting one more recipient and circumventing graft size discrepancy in one go. The first series was reported by Broelsch in 1990[10].

BIRTH OF LIVING DONOR LIVER TRANSPLANTATION

Experience gained from in-situ donor hepatectomy in reduced-size and split-graft DDLT paved the way for LDLT, an idea proposed by Smith as early as 1969[11]. When harvesting was performed on the living donor, much more technical ingenuity was required. The first attempt was made by Raia[1] and first success achieved by Strong of Australia[12] in July 1989. Under stringent review and auspices of the internal review board[13], the Chicago group led by Broelsch developed the first adult-to-child LDLT program[10]. Small series of adult-to-child LLDT were then reported from the United States[14] and Europe[15].

The problem of deceased donor liver graft shortage has been particularly severe in Asia[16]. In Japan, where deceased donor graft donation was non-existent[17] and liver surgery already well-developed, LDLT flourished[18,19]. For adult-to-adult LDLT (ALDLT), the left liver was used initially and was reported by the Shinshu group[20]. The left lobe used for adults was very often handicapped by the inadequate graft size. In 1993, Kyoto reported their improvisation of using the right lobe in a case of adult-to-child LDLT for a 9-year old recipient. The intention in this particular case was to avoid precarious arterial anatomy of the donor's left lobe[21]. The first case of right lobe ALDLT was performed at Queen Mary Hospital, the University of Hong Kong on May 10th 1996. *A priori*, the right liver

graft design included the middle hepatic vein (MHV). This was to address the problem of small-for-size syndrome[22]. The first series was reported shortly[23].

Donor right hepatectomy is one of the most major surgical living donor procedures. Subjecting a donor who has no medical indication for surgery to a major surgical operation with attendant risks is an ethical challenge. It was viewed by the medical community and the society with caution and skepticism[24-26]. Such donor procedure could only be partially justified by the benefit on the recipient and exhaustion of alternatives. This view is not universally accepted. Our common ground is the commitment to provide care of the highest standard to the living liver donor. Efforts for the betterment of care for the donors and yet not depriving them of the chance of saving or improving the life of their beloved recipients should worth dedication and ingenuity of the transplant community.

DONOR SAFETY AND WELLBEING

Donor safety is central to LDLT. As the application of LDLT extended from children to adults, and from using the left liver graft to the right liver graft, the dilemma between recipient success and donor risk came to the spotlight. The reported overall complication rate of donors is around 20%, but as high as 67% in one review[27]. A unified system of complication reporting[28] may narrow this range. Not only does the complication rate vary amongst different centers, the types of complications reported also vary. The most common complications are wound infection, ileus, and bile leakage. With accumulation of experience, donor morbidity could be lower than 20%. The majority of complications are of Grade I and were wound infections. With careful attention to biliary anatomy and guidance from intraoperative cholangiography, biliary complications are avoidable[29]. While one donor mortality is too many for the transplant community, there are already 14 known donor deaths[30]. Donor right hepatectomy carries a 0.5% donor mortality rate[30]. Similarly, the causes of donor mortality also vary[31]. A widely publicized case is a male donor in New York who succumbed to gas gangrene of *Clostridium perfringens* 3 d after donor right hepatectomy[32]. A hypertensive lady in Japan died from liver failure after right liver donation with a residual left liver with nonalcoholic steatohepatitis 28% of the total liver volume[33]. Fatal pulmonary embolism also occurred in a left liver donor[34]. A donor mother with a history of substance abuse also died from drug overdose 2 mo after donation to her 3 year-old son[35]. In other words, achieving a five-year recipient survival of 80%, it takes one donor life to save 160 recipients. Less tangible is the quality of life changes of the donor in comparison to the predonation state. The long-term biological consequences of donor hepatectomy are not fully known. Nevertheless, there are demonstrable drops in white cell count, platelet counts and elevation of liver transaminases even two years after right liver donation[36]. Quantification of such is mandatory in defining the field strength of LDLT. Detail of the holistic care of living liver donors deserves elaboration in a separate synopsis.

As agreed by the liver transplant community, living liver donors should be of good health[30] and the donor operation performed by experienced centers[37]. There should be no compromise of accepting potential donors with suboptimal physical and mental health. This is the only way to maintain or decrease donor mortality and morbidity.

RECIPIENT SHORT-TERM OUTCOMES

Graft size

Recipient survival is dependent on adequate graft size in relation to recipient body size[38]. Pathophysiology of the small-for-size graft and small-for-size syndrome is then defined[39]. Features include hepatocyte ballooning, steatosis, centrilobular necrosis, and parenchymal cholestasis. Pre-existing portal hypertension of the recipient increases the size requirement of graft[40].

Anecdotal success of using a very small graft for ALDLT 25% of the estimated standard liver weight[41] and even 20% with portosystemic shunting[42] had been reported. The paradigm shift from the left liver to the right liver enables adult recipients to undergo LDLT[43]. With technical maturity, 35% of the estimated standard liver weight remains the minimum requirement of a graft for predictable recipient success[44]. Portal hyperperfusion[45] and portal hypertension[46] are now conceived as possible mechanisms conducive to damage of small-for-size grafts. A battery of techniques for alleviation of portal venous flow was described. This includes superior mesenteric vein to mesocaval shunt[47], hemiportocaval shunting[48], inflow modulation by splenic artery ligation[49]. With portosystemic shunting using a saphenous vein interpositional graft between the right portal vein and right hepatic vein stump, a left lobe 20% of the estimated standard liver mass had been transplanted successfully in one patient[42]. Pharmacological manipulation is on the horizon as well[50].

More basic to these is the accurate assessment of standard liver volume of the recipient and thus the minimum graft size requirements. There have been a number of formulae developed from the west[51,52] and one from Japan[53]. A formula derived from Chinese and for application in Chinese which is also gender dependent has been developed and for validation[54].

Middle hepatic vein

Center to the controversy of right lobe ALDLT is inclusion of the MHV or otherwise. Deleterious effects of no drainage to the segments 5 and 8 include severe venous congestion and necrosis of these segments[55]. Surgical decision of not including the MHV includes demonstration of collaterals between segment 5 and 8 tributaries and the right hepatic vein[56]. Kyoto University devised an algorithm which includes the MHV when the graft is MHV dominant, or the graft to recipient weight ratio less than 1%, and in all cases, remnant left lobe larger than 35%[43]. Chang Gung Memorial Hospital includes the MHV when the graft to estimated standard liver volume is 50% or less, or when segment 5 and 8 hepatic veins are large and the right hepatic vein small[57]. Tokyo University ingeniously observed congestion of segments 5 and 8 of the graft after temporary clamping of the right hepatic artery before determining venous interpositional grafting[58].

We include the MHV in all right liver grafts for simplicity and familiarity of the technique[59]. Irrespective of the venous drainage pattern of segment 4 of the remnant left liver, the segment 4b hepatic vein is preserved. Utmost care is needed for its preservation when it drains into the MHV[60]. The outflow capacity is guaranteed by venoplasty on the back-table of the MHV and right hepatic vein into a single cuff[61]. The venoplasty is further marked by a more expedient hepatic vein to inferior vena cava anastomosis and higher outflow capacity of the right liver graft[62].

In summary, adequate graft size and quality, excellent venous outflow, and moderate portal inflow are keys to success of ALDLT[63].

High urgency LDLT

ALDLT under high urgency was impetus to development of liver transplant in our center[23]. Early experience of a number of centers showed inferior surgical outcomes of ALDLT in the high urgency situation[64-66]. With accumulation of experience and right liver graft incorporating the MHV, surgical outcomes of ALDLT is not compromised[67]. We also showed that ALDLT improves the survival of potential recipients[68]. The question of when a patient becomes too sick for liver transplantation is to be answered[69]. To justify ALDLT, good recipient outcome and acceptable donor morbidity, and voluntarism of the donor are the least that could be expected. The Live Organ Donor Consensus Group has largely supported this viewpoint[70]. The Model for End-Stage Liver Disease score has been validated as a factor predictive of recipient short-term survival in DDLT[71]. Data from ALDLT in North America[72] and Europe[73], however, do not support this view. Outside Asia, ALDLT is gradually considered a standard treatment for acute liver failure.

Biliary reconstruction

Biliary complication justifies itself the Achilles' heel of DDLT and is even more convincing in ALDLT[67]. Hepaticojejunostomy and duct-to-duct anastomosis have no substantial difference in the incidence of biliary complications. Nonetheless, duct-to-duct anastomosis reduces the operating time and avoids contamination of the operation field, expedites return of bowel functions, and avoids internal herniation of bowel loops. It also allows subsequent intervention by endoscopic retrograde cholangio pancreatography. In some centers, duct-to-duct anastomosis is stented to minimize the chance of stensosis and leakage. Whether the stent plays a role in the postoperative period, or in facilitating anastomosis, or both, has not been validated. Furthermore, whether continuous or interrupted sutures makes a difference is unknown. A study on DDLT which showed no difference[74] may not be applicable to ALDLT. Randomized controlled trials of recipients allocated to both arms may answer these questions.

RECIPIENT LONG-TERM OUTCOMES

Hepatocellular carcinoma

Early efforts of transplanting patients with advanced unresectable primary liver cancers were tempered by invariable relapse of malignancy[75]. Further work of the same group established the correlation between poor prognosis and high pathological tumor-node-metastasis staging[76]. Vascular invasion by tumor is the single most important factor in treatment failure of ALDLT for unresectable small hepatocellular carcinoma (HCC). Major vascular invasions though apparent for large tumors, may not be so for the small ones. Now called the Milan Criteria[77] and the University College of San Francisco Criteria (UCSF)[78], the tumor size and number are used as surrogate parameters for likelihood of vascular invasion. The Milan criteria are based on pretreatment imaging, whereas the UCSF criteria on liver explant histopathology. Accuracy of preoperative imaging in staging is inadequate[79]. The tendency is toward underestimation of tumor load. Even in studies with good image to histopathology correlation, underestimation is common. Tumor grade[80] and tumor size[81,82] are predictors of vascular invasion. Tumor size itself is also a predictor of tumor grade[81,82].

In our own series of ALDLT for HCC, there is a tendency of a higher recurrence rate compared with DDLT. It is postulated that the higher regeneration rate and reperfusion injury of small grafts in ALDLT provides an environment favorable for HCC cell implantation and growth in the graft[83]. It is also possible that in ALDLT, for preservation of the inferior vena cava, more liver manipulation is required leading to tumor compression and cancer cell dissemination. However, it is not unlikely that patients who have received DDLT are the self-selected patients because only candidates with slowly growing HCC who could wait for deceased donor liver grafts could receive the transplantation as the cancer cells are less aggressive. In fact, fast-tracking ALDLT for HCC had a higher recurrence rate[84]. Further studies on patient selection criteria and innovation of surgical technique are required to improve the long-term outcome of ALDLT for HCC. A recent series from Korea, nonetheless, has comparable results as DDLT[85].

In a series of 316 recipients with HCC who underwent ALDLT in Japan, the patient and recurrence-free survival rates were significantly worse if the Milan's criteria were not met. However, within this series, 171 (54.1%) of the recipients did not fulfill the Milan's criteria, and 176 were staged IVa. The alpha-fetoprotein level, tumor size, vascular invasion, and bilobar distribution were independent risk factors for HCC recurrence. The grade of histological differentiation of HCC showed close correlation with tumor characteristics and recurrence. Multifocal HCC verified by histopathology after transplantation with no recurrence was reported[86]. A policy of extended indication beyond the Milan and UCSF criteria is being validated[87].

The first use of sirolimus in liver transplantation was in patients with liver cancer, using the reported antitumor effects of the drug[88]. Subsequent work has shown efficacy of the drug in the inhibition of hepatocellular tumor cell lines[89,90]. Recipients transplanted for HCC and received sirolimus and low dose tacrolimus survived longer[91].

Sequelae of long-term immunosuppression

Calcineurin inhibitors greatly improve graft and recipient

Table 1 Landmark publications of liver transplantation in chronological order

Author	Discovery and application	Year of publication
Starzl[4]	First attempts of DDLT in human (1963)	1963
Starzl[5]	First long survival DDLT recipients (1967-1968)	1968
Smith[11]	Conceptualization of LDLT (1969)	1969
Calne[6]	Cyclosporin A used in solid organ transplantation	1979
Bismuth[8]	First adult-to-child reduced-size DDLT (1981)	1984
Pichlmayr[9]	Split-graft DDLT for 2 recipients (1988)	1988
Belzer[7]	Clinical use of University of Wisconsin Solution	1990
Raia[1]	First attempt of LDLT (December 1988)	1989
Strong[12]	First successful LDLT from adult to child (July 1989)	1990
Yamaoka[21]	First report of right liver graft from adult to child (1992)	1994
Hashikura[20]	First successful left liver adult-to-adult LDLT (November 1993)	1994
Lo[22]	First right liver adult-to-adult LDLT using right liver (May 1996)	1997

survivals. The lowest permissible serum drug level is employed to minimize the side effects. Nonetheless, two undesirable results still occur in recipients, i.e. renal impairment and tendency toward diabetes. A trend toward steroid-sparing immunosuppression seems workable[92]. The added benefit of the potential antineoplastic property of sirolimus makes it very attractive for recipients with renal impairment transplanted for resectable HCC[93].

Prompted by development of immune tolerance of noncompliant liver recipient after cessation of immunosuppressant therapy, weaning programs were incorporated into a long-term strategy of liver transplant programs. Drug-free tolerance was observed more frequently in humans after transplantation of the liver than of any other organs. Clinical application of cyclosporine[6] and then tacrolimus[94] dramatically prevented acute rejection of transplanted organs. However, drug-free tolerance became rare with the dominance of multiagent prophylactic immunosuppression[95].

Development of tolerance to the graft obviates the use of immunosuppressant with the side effects. The equivalence of solid organ and bone marrow transplantation is substantiated by documentation of systemic microchimerism[96]. Liver cells were identified in distant organs and host cells were also identified in liver grafts. The mirror image of solid organ and bone marrow transplantation envisioned by Starzl brings to light the practicality of long-term donor specific tolerance. The liver as a privileged graft[97] is a constant source of donor leukocyte, thus facilitates the process of clonal exhaustion-deletion especially in the early post-transplant phase under low immunosuppression pretransplant by antilymphocyte globulin. The concept of prope tolerance is also proposed as a means to a similar condition at a lesser degree[98]. Utilizing a powerful lymphocyte-depleting antibody, Campath 1H, half-dose cyclosporine monotherapy became workable. During a window of opportunity for immunologic engagement (WOFIE), it is hypothesized that there is engagement of donor and recipient marrow cells. Not until the availability of transgenic xenografts, effective and specific immunosuppression remains the practical way to graft maintenance. Nonetheless, continual assessment of the risk of chronic subclinical rejection is necessary[99].

CONCLUSION

At a minimum, recipient success is high and donor risk low. This brings donor and recipient issues into a close relationship. Conceptually, it would be inappropriate to accept a higher risk for the donor simply because of the improvement of recipient outcome. It is nonetheless our common goal to improve the standard of recipient and donor operations. What the public should change is, however, the better acceptance of ALDLT in the face of better safety and success, while the effort to make more deceased donor grafts available is never be forgotten.

Now we have near perfect graft harvesting and implantation techniques. Excluding patients with prohibitive conditions, e.g. uncontrolled sepsis and poor cardiac conditions, the short-term success is predictable. We still require selecting patients with a low recurrence rate of HCC and hepatitis C after transplantation. A lower biliary complication rate is welcome and could only be reduced by better preservation of biliary vasculature on the donor and the recipient and careful anastomotic techniques.

Donor safety and recipient success are inseparable. While donor mortality is a reality, it is by lowering donor mortality and improving recipient survival the justification of LDLT becomes stronger.

Although the major interest of the liver transplant community was in ALDLT in the last decade, the success of ALDLT has been a result of the ground works laid since the sixties. Key publications documenting the major achievements in liver transplantation leading to the ever improving results of ALDLT are listed in chronological order in Table 1.

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TOPIC HIGHLIGHT

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Role of peroxisome proliferators-activated receptors in the pathogenesis and treatment of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is highly prevalent and can result in nonalcoholic steatohepatitis (NASH) and progressive liver disease including cirrhosis and hepatocellular carcinoma. A growing body of literature implicates the peroxisome proliferators-activated receptors (PPARs) in the pathogenesis and treatment of NAFLD. These nuclear hormone receptors impact on hepatic triglyceride accumulation and insulin resistance. The aim of this review is to describe the data linking PPARα and PPARγ to NAFLD/NASH and to discuss the use of PPAR ligands for the treatment of NASH.

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Key words: Non-alcoholic fatty liver disease; Peroxisome proliferators-activated receptors; Insulin resistance; Metabolic syndrome; Pharmacologic ligands

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BACKGROUND ON NAFLD/NASH

An estimated 30% of adults and 10% of children and adolescents in the United States have nonalcoholic fatty liver disease (NAFLD), defined as liver fat content exceeding 5% (Figure 1)[1-3]. Non-alcoholic fatty liver disease is associated with obesity, non-insulin dependent diabetes, and hypertriglyceridemia and represents the hepatic manifestation of the metabolic syndrome[4]. A subset of persons with NAFLD progresses to nonalcoholic steatohepatitis (NASH), consisting of hepatic steatosis accompanied by inflammation and fibrosis (Figure 1)[5]. Nonalcoholic steatohepatitis affects approximately 3% of the lean population and 19% of obese persons, making it the most prevalent cause of chronic liver disease in the country[6]. Moreover, NASH represents a progressive form of liver disease. Cirrhosis developed in 5% of patients with NASH in a community-based cohort and 20% of NASH patients in a referral population[7,8]. Nonalcoholic steatohepatitis accounts for up to 75% of cases of cryptogenic cirrhosis and patients with NASH and cirrhosis are at risk for hepatocellular carcinoma[9,10].

The pathogenesis of NASH is often conceptualized as a two-step process, consisting of hepatic triglyceride accumulation, followed by the development of oxidative stress and cytokine expression leading to steatohepatitis[11]. Multiple metabolic processes can result in hepatocellular triglyceride accumulation including: (1) Excess dietary intake. Dietary triglycerides are delivered to the liver in the form of chylomicrons. In addition, dietary calories stored in adipose tissue as fat represent a source of fatty acids and triglycerides that can be delivered to the liver in the form of lipoprotein particles and free fatty acids. (2) Increased rates of lipogenesis resulting from the *de novo* synthesis of fatty acids and triglycerides in the liver. (3) Decreased rates of β-oxidation of fatty acids in the liver. (4) Decreased rates of export of cholesterol esters and triglycerides from the liver as very low density lipoprotein (VLDL)[12]. As shown in Figure 2, the PPARs impact on multiple processes involved in lipid trafficking and metabolism.

Insulin resistance and hyperinsulinemia seem to be central to the development of NAFLD. Insulin resistance is associated with increased lipolysis and reduced postprandial uptake and storage of fatty acids in adipose tissue, leading to increased fatty acid flux to the liver[13]. In turn, increased liver fat content contributes to hepatic insulin resistance[14]. Hyperinsulinemia induces sterol regulatory element-binding protein-1c (SREBP-1c) expression and hyperglycemia activates carbohydrate response element binding protein (ChREBP), both of which increase hepatic fatty acid synthesis[15].

THE PPARS

PPARs play a key role in modulating hepatic triglyceride

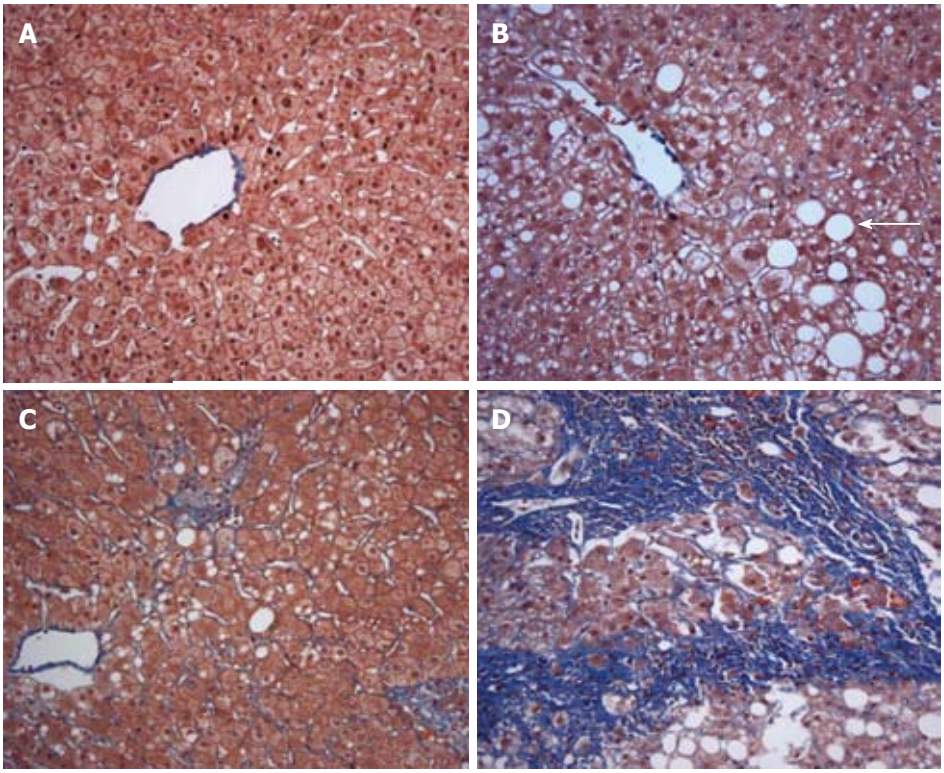


Figure 1 Liver histology ranging from normal liver to steatohepatitis with fibrosis. **A**: Normal liver. Cytoplasmic fat globules are absent in hepatocytes and there is no fibrosis in this trichrome stained specimen (× 20); **B**: Steatosis without steatohepatitis. Moderate cytoplasmic fat infiltration (arrow) is present without fibrosis (× 20); **C**: Steatohepatitis with minimal fibrosis. There is focal hepatocyte ballooning, inflammation, and minimal fibrosis (accentuated in blue by trichrome stain) (× 20); **D**: Steatohepatitis with fibrosis. There is nodular scarring in this fat laden liver with advanced fibrosis depicted in blue by trichrome stain (× 20).

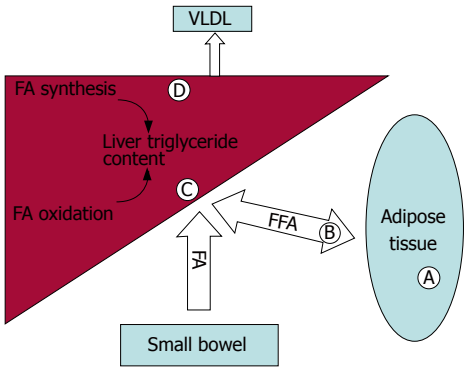


Figure 2 Mechanisms by which PPARs and their ligands can modulate triglyceride accumulation are highlighted by letters in the figure. A: PPARγ increases expression of genes associated with fatty acid uptake and triglyceride storage in adipocytes. Release of adiponectin from adipocytes improves insulin sensitivity and activates PPARα; B: PPARγ increases lipoprotein lipase expression, liberating circulating fatty acids from lipoproteins for import into adipocytes; C: PPARα activity up regulates β-oxidation of fatty acids in the liver; D: PPARα and TZDs upregulate stearoyl-CoA desaturase-1, a necessary enzyme for VLDL synthesis and export, and TZDs increase arachidonic acid content in triglycerides, which is associated with increased insulin sensitivity.

accumulation. PPARα regulates fatty acid β-oxidation. PPARγ increases insulin sensitivity as well as regulating triglyceride storage in adipose tissue. Fat labeling studies indicated that the majority of hepatic triglycerides originate from adipose tissue as non-esterified fatty acids[16].

PPARs are part of the nuclear receptor superfamily[17]. There are three isotypes in mammals designated PPARα [NR1C1], PPARδ [NR1C2] and PPARγ [NR1C3][18]. PPARα is activated by ligands termed peroxisome proliferators, which were named for their effects on peroxisomes in rodent livers[19,20]. Lipids are natural PPAR ligands, leading to regulation of lipid metabolism and fuel partitioning[17]. PPARs form a heterodimer with the retinoid X receptor (RXR). The PPAR:RXR heterodimer, when bound to a ligand, changes conformation and binds to DNA at PPAR response elements, resulting in gene transcription[21,22].

PPARα AND NAFLD

PPARα is expressed in the liver and other metabolically active tissues including striated muscle, kidney and pancreas[23,24]. Many of the genes encoding enzymes involved in the mitochondrial and peroxisomal fatty acid β-oxidation pathways are regulated by PPARα. In particular, the acyl-CoA synthetase, the carnitine palmitoyl transferase I, the very long-chain acyl-CoA dehydrogenase and the tri-functional protein genes encoding enzymes in the mitochondrial fatty acid β-oxidation pathway are induced by peroxisome proliferators that activate PPARα[25-28]. Similarly, the acyl-CoA synthetase, the straight-chain acyl-CoA oxidase, the L-bifunctional protein and the 3-ketoacyl-CoA thiolase genes encoding enzymes in the peroxisomal fatty acid β-oxidation pathway are induced by peroxisome proliferators that active PPARα[26,27,29,30]. Loss of expression of the PPARα gene in mice results in hepatic steatosis under conditions of increased fatty acid metabolism in the liver such as fasting or a high fat diet[31,32]. Administration of a potent PPAR agonist decreases hepatic steatosis in mice receiving a methionine and choline deficient diet[33]. These observations indicate that under conditions of increased hepatic fatty acid influx or decreased hepatic fatty acid efflux, PPARα activation prevents the accumulation of triglycerides by increasing the rate of fatty acid catabolism.

Additional factors appear to interact with PPARα to regulate hepatic triglyceride content. These include adiponectin, which is an adipocyte produced peptide

hormone that limits fat accumulation in the liver by a number of mechanisms including activation of PPARα to increase hepatic fatty acid oxidation[34]. In cell culture models, treatment with adiponectin resulted in increased activity of PPARα target genes such as acyl-CoA oxidase, carnitine palmitoyl transferase- I , and fatty acid binding protein[35]. PPARα ligands can increase steaoryl-CoA desaturase-1 (SCD1) activity, which is necessary for VLDL secretion[36]. A PPAR response element was found in the SCD1 promoter[37]. Adiponectin is upregulated by PPARγ, providing a connection between the two isotypes[38].

Most of the data regarding PPARα and hepatic lipid homeostasis comes from mouse models. However, there are important differences in PPARα activity between rodents and humans. PPARα DNA binding activity and PPARα expression in human hepatocytes is less than 10-fold that observed in mice[39,40]. Certain PPAR response elements, such as the acyl CoA oxidase gene, do not respond to PPAR ligands in humans as they do in rodent models[41]. Finally, PPARα activation in rodent models resulted in peroxisomal proliferation, hepatomegaly, and hepatocellular carcinoma[39,42,43], whereas similar changes were not observed in humans[44,45]. Further research is needed to determine the relative importance of PPARα in regulating hepatic triglyceride metabolism in humans.

PPARα AS A TARGET FOR THE TREATMENT OF NAFLD

Fibric acid derivatives, which are available for use in humans as lipid lowering agents, serve as PPARα activators[46,47]. In a mouse model of fatty liver disease, fenofibrate treatment improved steatosis and increased expression of genes involved in fatty acid metabolism[48]. Trials with fibrates in humans have yielded mixed results. A study involving potential living liver donors with steatosis showed that a combination of diet, exercise, and benzafibrate significantly reduced steatosis and resulted in normalization of alanine aminotransferase levels[49]. However, it was not clear whether the therapeutic benefit was related to benzafibrate or to a 1000 kilocalorie/day diet and a 600 kilocalorie/day exercise regimen. In addition to being a PPARα ligand, benzofibrate activates PPARγ and improves insulin sensitivity in animal models[46,47], an effect not seen with fenofibrate[50]. Another study demonstrated that 42% of 62 patients with NAFLD had biochemical and ultrasound improvement on fenofibrate, but histologic data were not collected[51]. A small controlled study of gemfibrozil *versus* placebo for four weeks found improved aminotransferase levels with the use of gemfibrozil in patients with NAFLD[52]. These studies are in contrast to a another small series, which demonstrated no change in aminotransferases and no histologic improvement after one year of clofibrate therapy for NAFLD[53].

Omega-3 polyunsaturated fatty acids (PUFA) present in fish oil, and their metabolites, provide another source of PPARα ligands. Omega-3 PUFA also inhibit lipogenesis by antagonizing activation of LXR[54,55], thus reducing expression of SREBP-1c[56], which results in the down regulation of key enzymes involved in hepatic lipid biosynthesis. In mouse models, omega-3 PUFA supplementation was associated with improvement in hepatic steatosis and insulin sensitivity, as well as lower fasting free fatty acid concentrations and lower serum triglyceride levels[57,58]. Two human studies reported a decline in serum aminotransferase levels and improvement in ultrasound features of fatty liver with omega-3 PUFA supplementation[59,60]. However, no histologic data were provided. Omega-3 PUFA supplementation also reduces serum triglyceride levels in the fasting and postprandial state[61-63], but was not found to improve insulin sensitivity in humans[62,64,65].

PPARγ AND NAFLD

PPARγ is expressed in high levels in adipose tissue[66] and plays a role in increasing insulin sensitivity as well as in promoting fatty acid uptake into adipocytes and adipocyte differentiation. The net effect of these processes is to increase triglyceride storage in adipocytes, reducing delivery of fatty acids to the liver. Patients with dominant negative mutations in PPARγ have NAFLD and the metabolic syndrome while lacking adipose tissue suggesting increased triglyceride delivery to the liver[67]. PPARγ is present in the liver to a lesser degree than in adipose tissue. Liver-specific PPARγ deficient mice are protected against the development of steatosis suggesting a role for hepatic PPARγ in liver triglyceride accumulation[68,69].

Insulin resistance is integral to the development of NAFLD, leading to increased fatty acid flux to the liver and increased hepatic fatty acid synthesis[13,15]. PPARγ increases insulin sensitivity by upregulating GLUT4, an insulin dependent glucose transporter in adipose tissue and striated muscle[70], and inducing expression of the c-Cbl associated protein, which is involved in insulin signaling[71]. Additionally, in mouse models of insulin resistance, PPARγ activation attenuated induction of supressor of cytokine signaling 3 (SOCS3), which is involved in the development of insulin resistance[72].

PPARγ also promotes adipocyte differentiation and expression of proteins in adipocytes involved in fatty acid uptake[17,73], fatty acid transport[74,75] and fatty acid synthesis[76]. Differentiation of preadipocytes to adipocytes requires transcription factors including the CCAT-enhancer-binding proteins (C/EBPs) and the adipocyte differentiation and determination factor (ADD)-1/SREBP-1[77-80]. C/EBP plays an important role in inducing and maintaining PPARγ expression in adipogenesis[81,82]. ADD-1/SREBP-1 is strongly adipogenic, is enhanced by PPARγ, and results in the expression of lipogenic genes including fatty acid synthase[80]. These transcription factors guide the cell through proliferation, clonal expansion, growth arrest, and eventually adipocyte specific genes are activated resulting in lipid accumulation[82]. PPARγ also increases expression of lipoprotein lipase, an enzyme that serves to partition fat to adipocytes, limiting fatty acid flux to the liver. Similar to PPARα, PPARγ ligands upregulate SCD1 activity, which promotes VLDL secretion. Thiazolidinediones (TZDs), ligands for PPARγ have also been shown to increase arachidonic acid content in triglycerides through SCD1, which has been associated with increased

insulin sensitivity[83]. Other effects of PPARγ include induction of uncoupling protein-2, which might decrease hepatic triglyceride accumulation by increasing energy expenditure[84]. PPARγ expression also might reduce hepatic inflammation by decreasing expression of proinflammatory cytokines, such as TNFα[85].

PPARγ AS A TARGET FOR THE TREATMENT OF NAFLD

TZDs are PPARγ agonists, which improve glycemic control in patients with type 2 diabetes mellitus by increasing insulin sensitivity[86]. The TZD-mediated increase in insulin sensitivity was demonstrated in adipose tissue, the liver, and skeletal muscle[87,88]. TZD therapy increases adiponectin levels, which are associated with improved insulin sensitivity[89]. Furthermore, adiponectin impacts on hepatic fat accumulation by enhancing fatty acid oxidation in muscle, and by activating PPARα to increase fatty acid oxidation in the liver[34].

Thiazolidinediones also increase expression of AMP-activated protein kinase[88,90]. This protein kinase increases fatty acid oxidation as well as decreasing lipogenesis[91,92]. The reduction in lipogenesis is mediated through phosphorylation and inhibition of acetyl-CoA carboxylase, which decreases malonyl CoA formation and down regulates SREBP and the carbohydrate response element binding protein (ChREBP)[93]. Finally, TZDs have anti-inflammatory and anti-fibrotic properties that might be beneficial in NASH. Serum high-sensitivity CRP, IL-6 and IL-18 levels were significantly reduced in patients on TZD therapy[94,95] and the TZD pioglitazone reduced activation of hepatic stellate cells in an animal model[96]. Increased adiponectin may also contribute to the anti-inflammatory effects of TZD therapy. Adiponectin was shown to block TNFα activation of inflammatory genes in endothelial cells[97], decrease macrophage growth and function[98-100], and increase release of the anti-inflammatory cytokines IL-10 and IL-1RA with a concomitant decrease in interferon-γ production[100].

Studies of the TZDs rosiglitazone and pioglitazone demonstrated reduction in aminotransferase levels and improvement in liver histology in patients with NASH[87,101-106]. One study that compared pioglitazone plus vitamin E to vitamin E alone for the treatment of NASH found significant improvement in steatosis, hepatocellular ballooning, and pericellular fibrosis in the combination therapy arm, but not in patients treated with vitamin E alone[103]. In a study of pioglitazone plus diet versus placebo plus diet in patients with biopsy proven NASH and insulin resistance, pioglitazone therapy was associated with a significant reduction in mean serum aminotransferase levels and improved glycemic control[107]. There were significant improvements in hepatic insulin resistance as well as histologic parameters including hepatic steatosis, ballooning, and inflammation, although not fibrosis with six months of treatment. Further evaluation of the efficacy and the cardiovascular risk of TZD therapy[108] is needed before this class of medications is routinely prescribed for the treatment of NASH.

CONCLUSION

The nuclear hormone receptors PPARα and PPARγ appear to play an important role in modulating hepatic triglyceride accumulation, the primary process in the development of NAFLD. PPARα activity reduces liver fat by increasing β-oxidation of fatty acids and PPARγ increases insulin sensitivity as well as reducing fatty acid flux to the liver. PPAR ligands show promise in the treatment of NAFLD, although further human studies are needed to define the therapeutic role of these agents.

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BASIC RESEARCH

Effect of notoginsenoside R1 on hepatic microcirculation disturbance induced by gut ischemia and reperfusion

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Abstract

AIM: To assess the effect of notoginsenoside R1 on hepatic microcirculatory disturbance induced by gut ischemia/reperfusion (I/R) in mice.

METHODS: The superior mesenteric artery (SMA) of C57/BL mice was ligated for 15 min to induce gut ischemia followed by 30-min reperfusion. In another set of experiments, R1 was continuously infused (10 mg/kg per hour) from 10 min before I/R until the end of the investigation to study the influence of R1 on hepatic microcirculatory disturbance induced by gut I/R. Hepatic microcirculation was observed by inverted microscopy, and the vascular diameter, red blood cell (RBC) velocity and sinusoid perfusion were estimated. Leukocyte rolling and adhesion were observed under a laser confocal microscope. Thirty and 60 min after reperfusion, lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate transaminase (AST) in peripheral blood were determined. The expression of adhesion molecules CD11b/CD18 in neutrophils and tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) in plasma were evaluated by flow cytometry. E-selectin and intercellular adhesion molecule-1 (ICAM-1) in hepatic tissue were examined by immunofluorescence.

RESULTS: After gut I/R, the diameters of terminal portal venules and central veins, RBC velocity and the number of perfused sinusoids were decreased, while the leukocyte rolling and adhesion, the expression of E-selectin in hepatic vessels and CD18 in neutrophils, IL-6, MCP-1, LDH, ALT and AST were increased. R1 treatment attenuated these alterations except for IL-6 and MCP-1.

CONCLUSION: R1 prevents I/R-induced hepatic microcirculation disturbance and hepatocyte injury. The effect of R1 is related to its inhibition of leukocyte rolling and adhesion by inhibiting the expression of E-selectin in endothelium and CD18 in neutrophils.

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Key words: Ischemia/reperfusion; Notoginsenoside R1; Leukocytes adhesion; E-selectin; Hepatic injury

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INTRODUCTION

It is well recognized that gut ischemia/reperfusion (I/R) induces injury of distant organs, such as liver and lung[1-3]. A rate-limiting step in the pathogenesis of I/R injury of liver and other organs is the recruitment of leukocytes to vascular endothelium[2,4,5]. Oxygen free radicals produced by gut I/R activate nuclear factor kappa-B (NF-κB)[6,7], initiate expression of selectin and adhesion molecules[8-10], and elicit release of proinflammatory mediators like tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)[10]. The expression of L-selectin on leukocytes and E-selectin on endothelial cells induces the rolling of leukocytes along the vascular endothelium[11,12], which further promotes the expression of adhesion molecules CD11b, CD18 on

leukocytes and intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, resulting in adhesion of leukocytes to vascular endothelial cells[2,4,13,14]. In addition, proinflammatory mediators produced by I/R enhance the expression of selectins and adhesion molecules, and aggravate the rolling and adhesion of leukocytes[8,15]. The adhesion of leukocytes to the vascular endothelium results in release of oxygen free radicals and proteinase, thus inducing hepatic injury[16]. In line with these findings, studies indicate that inhibition of the expression of selectins and adhesion molecules on leukocytes and endothelial cells ameliorates the adhesion of leukocytes to the hepatic vascular endothelium after gut I/R and attenuates hepatic microcirculation disturbance and injury[2,4,17].

Panax notoginseng (PN) is the dried root of *Panax notoginseng* (Araliaceae), a Chinese herb medicine widely used in China, Korea, Japan and other Asian countries in the treatment of microcirculatory disturbance-related diseases, such as cardiovascular disease, cerebral vascular diseases and liver dysfunction[18,19]. PN contains more than 30 different types of saponin, of which ginsenoside Rg1 (Rg1), ginsenoside Rb1 (Rb1) and notoginsenoside R1 (R1) are the eminent members[20]. Previous studies have proved that *Panax notoginseng saponins* (PNS) improve I/R-induced hepatic microcirculation disturbance[19], inhibit platelet aggregation and adhesion molecule expression, and improve vascular endothelium function[21]. It was also reported that PNS inhibit adhesion of leukocytes to rat mesentery venules and expression of neutrophil adhesion molecules CD11b and CD18 induced by lipopolysaccharide (LPS)[22]. The expression of LPS-induced vascular endothelial TNF-α is inhibited by R1 by inhibiting degradation of the inhibitor kappa-B (I-κB)[23]. It has been shown that cardiotonic pills (CP, a traditional Chinese medicine containing PN, *salvia miltiorrhiza* and *borneol*) can inhibit adhesion of leukocytes to the hepatic vascular endothelium in rats induced by gut I/R and chronical ethanol feed, and blunt the concentration increment of peripheral blood alanine aminotransferase (ALT), TNF-α and LPS[24]. However, whether R1 improves gut I/R-induced hepatic microcirculation disturbance has not yet been reported, although it is highly anticipated as a major component of PNS, and the structure of R1 has been identified (Figure 1). Therefore, by virtue of intravital microscopy the present study explored the dynamic effects of R1 on a mouse hepatic microcirculation disturbance model induced by gut I/R, especially on leukocyte rolling and adhesion on the vascular endothelium. The expression of E-selectin and ICAM-1 was also determined by immunofluorescent technique. The expression of neutrophil adhesion molecules CD11b and CD18, and the concentration of proinflammatory mediators such as TNF-α, IL-6 and monocyte chemotactic protein-1 (MCP-1) was measured by flow cytometry. The concentrations of lactate dehydrogenase (LDH), ALT and aspartate aminotransferase (AST) were also determined.

MATERIALS AND METHODS

Notoginsenoside and reagents

R1 (purity > 98%) was supplied by Tianjin Talsy Group (Tianjin, China). Other reagents used in experiments were as follows: rhodamine 6G (purity > 99.0%, Lot No.2350994, Fluka Co., Switzerland), FITC-rat anti-mouse CD18 monocolonal antibody (Lot No.553293, BD Biosciences PharMingen, USA), FITC-rat anti-mouse CD11b monoclonal antibody (Lot No.557396, BD Biosciences PharMingen, USA), goat polyclonal antibody against mouse E-selectin (M-20) (sc-6939, Santa Cruz Biotechnology, Inc. USA), goat polyclonal antibody against mouse ICAM-1 (M-19) (sc-1511, Santa Cruz Biotechnology, Inc. USA), rhodamine conjugated rabbit anti-goat lgG-R (Lot No.B1006, Santa Cruz Biotechnology, Inc. USA), Hoechst33342 (Lot No.6538, Santa Cruz Biotechnology, Inc. USA), mouse MCP-1 flex set (Lot No.558342, BD Biosciences, USA), mouse TNF flex set (Lot No.558299, BD Biosciences, USA), mouse IL-6 flex set (Lot No.558301, BD Biosciences, USA).

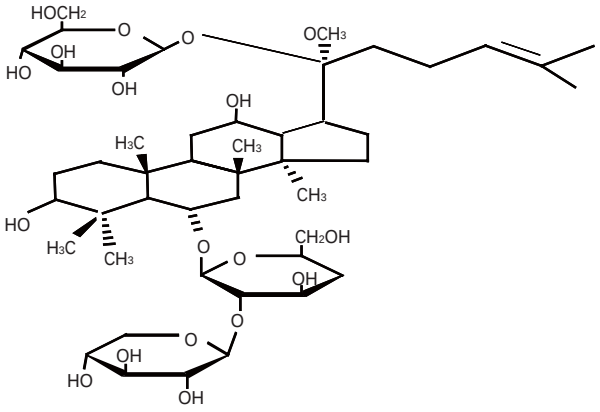


Figure 1 Chemical structure of notoginsenoside (R1).

Animals

C57/BL mice, weighing 22-26 g and aged 8-10 wk (the animal certificate number was SCXK 2002-2001) were purchased from the Animal Center of Peking University Health Science Center. The animals were caged at 24℃ ± 1℃ with a humidity of 50% ± 5% in a 12 h light/dark cycle, and starved with free access to water for 12 h before the experiment. All animals were handled according to the Guidelines of the Peking University Animal Research Committee.

Intravital microscopy

C57/BL mice were anesthetized with 20% urethane (10 mL/kg body wt, im), as previously described[25]. The left jugular vein was cannulated for drug administration with a polyethylene pipe (0.96 mm in diameter). Immediately after laparotomy, the mice were placed on an observation board in lateral position. The liver was placed on an adjustable Plexiglas microscope stage within a thermo-controlled (37℃) observation box and carefully handled to minimize the influence of respiratory movements. The left lateral lobe of liver was observed under an inverted intravital microscope (DM-IRB, Leica, Germany) assisted by a 3CCD colour camera (JK-TU53H, 3CCD camera, Toshiba, Japan). Areas (400 μm × 320 μm) were selected that included both terminal portal venules

and central veins without adherent leukocytes for observation. Images of the microcirculation of liver surface were monitored through a × 20 objective, and the dynamics of hepatic microcirculation was recorded on DVD discs using a DVD recorder (DVR-R25, Malata, China). The liver surface for observation was moisturized with 37℃ physiological saline drops throughout the whole procedure, and the liver surface around the observation region was covered with saline-soaked cotton gauze[26].

Procedure for ischemia and reperfusion

The surface of liver was observed for 10 min before ligation of the superior mesenteric artery (SMA) to ensure that all parameters measured were in a steady state. The SMA was then ligated with a snare created from polyethylene tubing (1.00 mm) for 15 min. After ischemia, the ligation was gently released for reperfusion. Venular diameter, RBC velocity, sinusoidal reperfusion, and leukocyte rolling and adhesion were determined immediately before ischemia (baseline) and every 15 min after reperfusion for half an hour.

Experimental protocols

Mice in the I/R group were continuously infused with vehicle saline through the jugular vein from 10 min before I/R until the end of the observation. Mice in the R1 + I/R group were continuously infused with R1 (10 mg/kg per hour) from 10 min before I/R until the end of the observation. Mice in the sham-operated control group were treated in an identical fashion as those in the I/R group, but not subjected to ligation of the SMA. Six mice (three males, three females) were included in each group.

Determination of microcirculatory parameters

The diameters of terminal portal venules and central veins were on replayed DVD images using Image-Pro Plus 5.0 software[27]. The result was presented as the ratio of the value determined at 15 min or 30 min to the baseline.

To access the sinusoidal perfusion, the number of hepatic sinusoids with red blood cells (RBCs) flowing through in the hepatic terminal portal venule and central vein regions was scored on the DVD replay, and presented as the perfused hepatic sinusoids/field of view (250 μm × 300 μm)[26]. The result was presented as the ratio of the value determined at 15 min or 30 min to the baseline.

The RBC velocity in hepatic terminal portal venule and central vein was recorded at a rate of 1000 frames/s by changing the monitor from CCD to a high speed video camera system (FASTCAM-ultima APX, photon, Japan), and the recordings were replayed from the high speed stored images at a rate of 25 frames/s. The RBC velocity in venules was measured with Image-Pro Plus 5.0 software[26,27]. The velocity was presented as μm/s. The result was presented as the ratio of the value determined at 15 min or 30 min to the baseline.

To evaluate the leukocyte rolling and adhesion the fluorescence tracer 0.2 mL Rhodamine 6G (0.5 mg/mL in physiological saline) was administrated *via* the left jugular vein for the selective staining of white blood cells *in vivo*[28-32]. Under the inverted laser confocal microscope system (BIO-RAD, Radiance 2100, A xiovert 200, Carl Zeiss Shanghai Co, Ltd, German), with 20 × fluorescent object lens, irradiated with the argon laser beam (wavelength = 543 nm), the rolling and adhesion of leukocytes in hepatic terminal portal venules and central veins were recorded. At each time point, a total of 10 successive frames were recorded at a scanning speed of 1 frame/s, and adhered leukocytes were defined as those that appeared at the same position in the 10 successive frames[24]. The adhered leukocytes in hepatic terminal portal venules and central veins were presented as the number of leukocytes/200 μm, while those within the hepatic sinusoid were counted as the number per field of view of 200 μm^2. The leukocytes that stayed at the same position in hepatic terminal portal venules and central veins for less than 10 s were designated as rolling leukocytes, and presented as the number/200 μm.

Analysis of immnofluorescent staining of hepatic endothelial adhesion molecules E-selectin and ICAM-1

After 30-min reperfusion, liver was fixed with 4% paraformaldehyde perfusion, removed and frozen with liquid nitrogen, then cut into sections of 6 μm by a cryostat (LEICA CM 1800, Leica Co., German). The sections were further fixed with 4% paraformaldehyde at room temperature for 10 min and washed with PBS. The samples were then immunohistochemically stained as routing. Goat polyclonal antibody against mouse E-selectin or goat polyclonal antibody against mouse ICAM-1 was applied at dilution of 1:50. The secondary antibody (Rhodamine-labeled rabbit anti goat IgG diluted at 1:200) was added and incubated at 37℃ for 30 min, followed by washing with PBS and incubation with Hoechst 33342 (2 μg/mL) at room temperature for 3 min[33]. After washed with PBS, the specimen was sealed and observed under Laser confocal microscope with 63 × object lens. Fluorescence intensity was detected at excitation wavelength 543 nm for R-phycoerythrin and 405 nm for Hoechst (nuclear staining). Five fields of view (1.6×10^4 μm^2 each) were evaluated in mouse hepatic sinusoids for each condition. Fluorescence intensities of E-selectin or ICAM-1 were estimated by Image Pro Plus software and expressed as an average proportion, positive area/area of one field of view (1.6×10^4 μm^2).

Assessment of the expression of adhesion molecules CD11b and CD18 in peripheral neutrophils

After 30-min reperfusion, blood was collected via inferior vena cava and anticoagulated with heparin (20 unit/mL whole blood). The sample was incubated with 1 μg FITC-labeled antibody against CD18 or CD11b for 20 min at room temperature in dark. RBCs were lysed by addition of hemolysin and the samples were then washed twice with PBS. Flow cytometry (FACS Calibur, B.D. Co, USA) was used to assess the mean fluorescence intensity of CD11b or CD18 for 5000 neutrophils in each condition[22].

Peripheral blood hepatic enzyme assay

In some mice, at 30 min and 60 min, respectively after reperfusion, blood samples were withdrawn *via* inferior vena cava and anticoagulated with heparin (20 unit/mL whole blood). The blood serum was isolated by

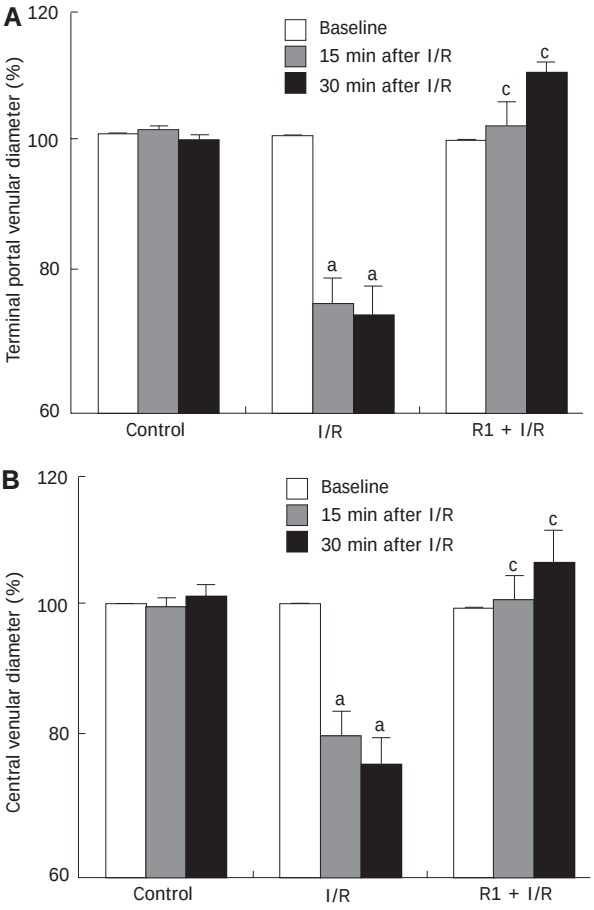


Figure 2 Effect of R1 on the terminal portal venular diameter (**A**) and central venular diameter (**B**) of hepatic venules of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the ratio of the diameter value at a time point to the baseline. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

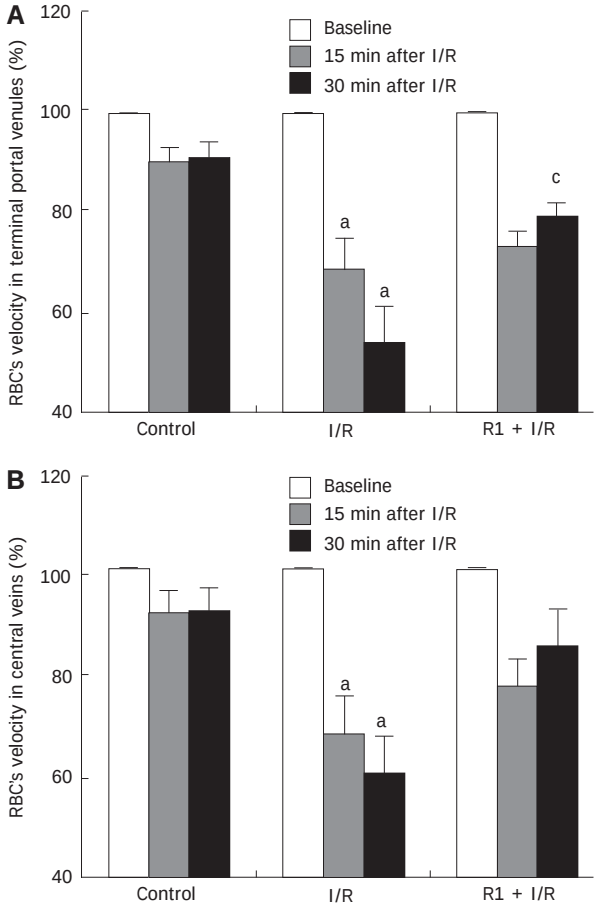


Figure 3 Effect of R1 on the RBC velocity in terminal portal venlues (**A**) and in central veins (**B**) of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the ratio of the RBC velocity value at a time point to the baseline. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

centrifugation (Allegra™ 64R Centrifuge, Beckman Coultertm, German) at 4000 r/min for 10 min at 4℃ and stored at -20℃. The activities of LDH, ALT, and AST were measured respectively using lactate dehydrogenase, alanine aminotransferase and aspartate aminotransferase kits with parameter rate-A[34], following their manufacturer's instructions, with an automatic enzyme analyzer (7170A Automatic Analyzer, Hitachi, Japan).

Peripheral blood TNF-α, IL-6 and MCP-1 assay

At 30 min after reperfusion, blood was collected *via* inferior vena cava, and anticoagulated with heparin (20 unit/mL whole blood). The blood serum was isolated by centrifugation (Allegra™ 64R Centrifuge, Beckman Coultertm, German) at 4000 r/min for 10 min at 4℃ and stored at -20℃. The concentrations of TNF-α, IL-6 and MCP-1 were measured by flow cytometry with a BD cytometric bead array kit (BD Biosciences Pharmingen, USA)[35]. Fifty μL bead was added into 50 μL blood plasma or standard substance and incubated at room temperature in dark for 1 h for bead capture. Fifty μL PE-labelled detecting antibody was then added and incubated at room temperature for 2 h to form a sandwich complex. After incubation, the samples were washed thoroughly with 1 mL washing buffer (BD Biosciences Pharmingen, USA). The mean fluorescence intensity of TNF-α, IL-6 and MCP-1 was detected respectively by flow cytometry (FACS Calibur, B.D. Co., USA) and the data were analyzed using the BD cytometric bead array analysis software.

Statistical analysis

Values are presented as mean ± SE ($n = 6$), *F*-test was performed using SPSS 10.0 statistical software. $P < 0.05$ was considered statistically significant.

RESULTS

The effect of R1 on the diameter of hepatic terminal portal venule and central vein of mice subjected to SMA I/R are shown in Figure 2. In the control group, the diameters of both terminal portal venules and central veins remained nearly constant over the entire observation period. SMA I/R decreased the diameter of vessels in a time-dependent manner, while treatment with R1 significantly relieved SMA I/R-induced decrease in the vessel diameters.

The influence of R1 on the RBC velocity in hepatic terminal portal venules and central veins of mice after SMA I/R is shown in Figure 3. In the control group, no significant change was observed in the RBC velocity of both

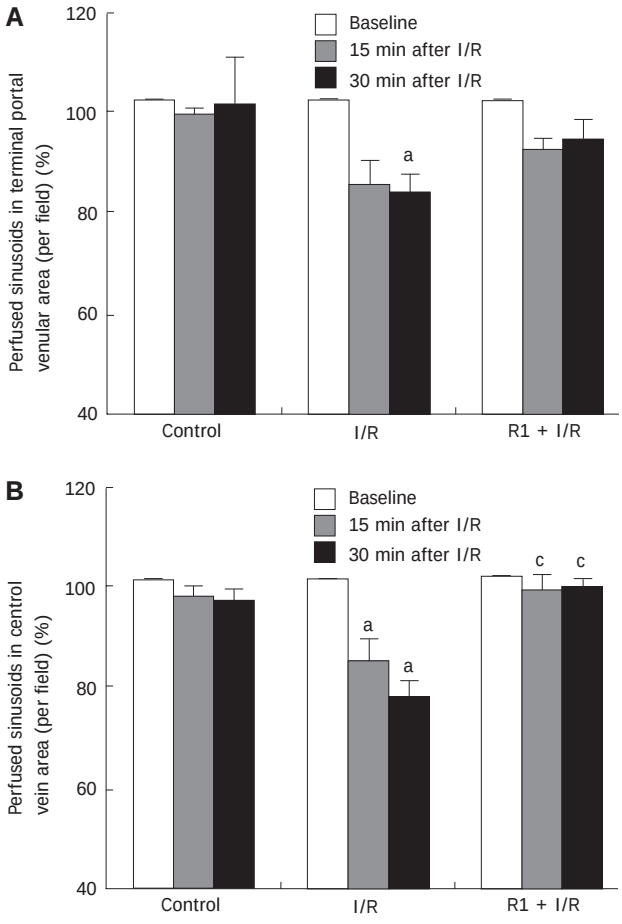


Figure 4 Effect of R1 on the perfused hepatic sinusoids in the areas of terminal portal venules (**A**) and in the area of central veins (**B**) of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the ratio of perfused sinusoids at a time point to the baseline. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

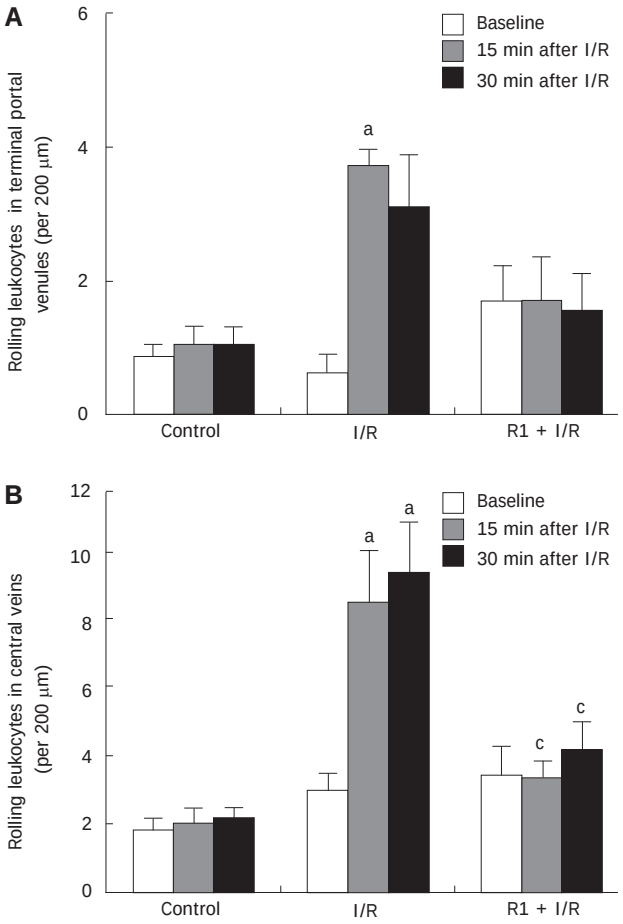


Figure 5 Effect of R1 on the rolling leukocytes in the areas of terminal portal venlues (**A**) and central veins (**B**) of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the number of rolling leukocytes per 200 μm. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

types of vessels during the period of observation. SMA I/R significantly decreased the RBC velocity of vessels in a time-dependent fashion. R1 treatment blunted the SMA I/R-induced decrease in the RBC velocity of both types of vessels at 30 min after reperfusion, being significant in terminal portal venules (Figure 3A) but not in central veins (Figure 3B), in comparison with the I/R group.

The effect of R1 on the number of reperfused sinusoids in hepatic terminal portal venule and central vein areas after SMA I/R is depicted in Figure 4. In the control group, no significant change in the number of reperfused sinusoids was detected either in the terminal portal venule area or in the central vein area through the entire observation. SMA I/R exposure elicited a time-dependent decrease in the number of reperfused sinusoids, which became statistically significant at 15 min of reperfusion in the central vein areas and at 30 min of reperfusion in the terminal portal venule areas compared to the control group. I/R-induced decrease in the number of reperfused sinusoids in central vein areas was attenuated significantly after treatment with R1 (Figure 4B), and this effect was not observed in the terminal portal venule areas (Figure 4A).

The effect of R1 on the number of rolling leukocytes in mouse hepatic terminal portal venules and central veins after SMA I/R is depicted in Figure 5. I/R challenge significantly increased the number of rolling leukocytes in both terminal portal venules and central veins when compared with the control group, although a small number of rolling leukocytes could be observed in the control group during the period of examination. R1 treatment reduced the enhancement in the leukocyte rolling induced by I/R, which was statistically significant in the central veins (Figure 5B), but not in terminal portal venules (Figure 5A).

Figure 6 shows the effect of R1 on the adhesion of leukocytes induced by SMA I/R. SMA I/R increased the number of adherent leukocytes in both hepatic terminal portal venule and central vein regions, which was attenuated after treatment with R1, being significant in the terminal portal venules (Figure 6A), but not in the central veins (Figure 6B).

The effect of R1 on the leukocyte adhesion in sinusoids of hepatic terminal portal venule and central vein areas was determined (Figure 7). As in the hepatic terminal portal venules and central veins, only a small number of adherent leukocytes were visualised within sinusoids either of either the terminal portal venule area or the central vein area in the control group. A remarkable increase in the number of adherent leukocytes within sinusoids of

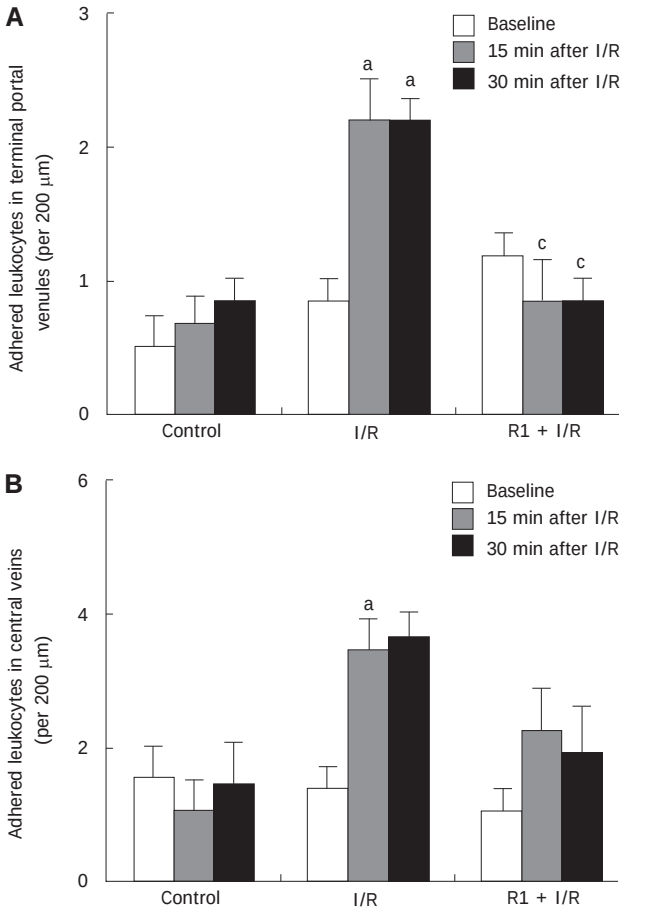


Figure 6 Effect of R1 on the adherent leukocytes in terminal portal venlues (**A**) and central veins (**B**) of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the number of adherent leukocytes per 200 μm. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

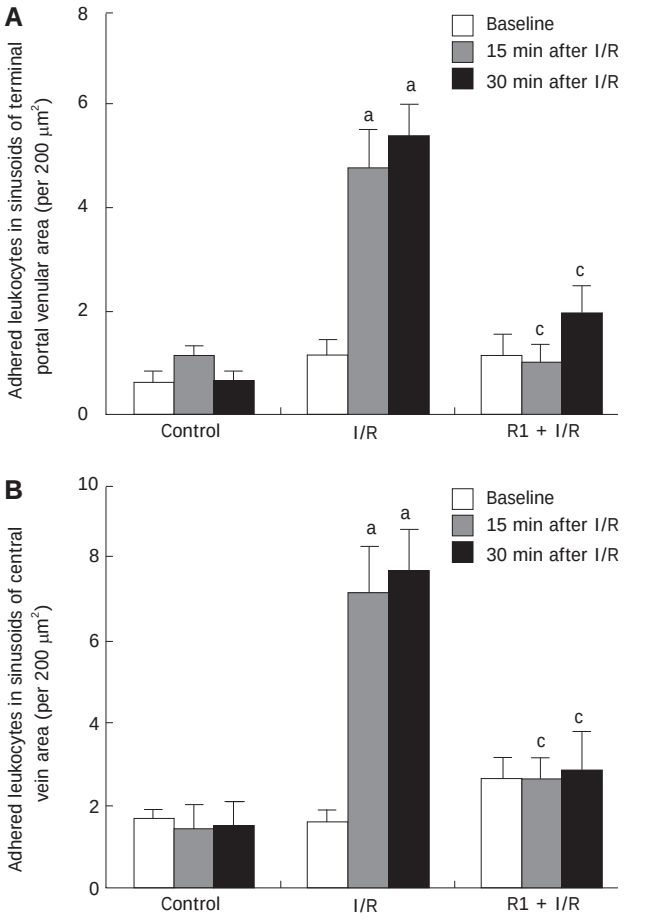


Figure 7 Effect of R1 on the adherent leukocytes in the hepatic sinusoids in the areas of terminal portal veins (**A**) and central veins (**B**) of mice subjected to SMA I/R at 0 min before ischemia (Baseline) and 15 min, 30 min after reperfusion. Abscissa represents the number of adherent leukocytes per field of view of 200 μm². The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

both areas was observed when the mice were subjected to SMA I/R, which was inhibited significantly after treatment with R1 (Figure 7) starting from 15 min after reperfusion.

Figure 8 shows the expression of E-selectin in mouse hepatic sinusoids in the sham, I/R and R1 + I/R groups after 30 min of reperfusion. Thirty minutes after reperfusion, the expression of E-selectin increased (Figure 8B) compared with the sham group (Figure 8A), and treatment with R1 (Figure 8C) suppressed the increase in I/R-elicited E-selectin expression.

The influence of R1 on the expression of E-selectin in mouse hepatic sinusoids after 30 min of reperfusion was quantitatively evaluated (Figure 9A). Thirty-minute reperfusion significantly enhanced the expression of E-selectin. The SMA I/R-induced increase in the expression of E-selectin was completely ablated after treatment with R1.

The expression of ICAM-1 in mouse hepatic sinusoids after 30 min of reperfusion had no significant change. Treatment with R1 had no significant influence on the expression of ICAM-1 either (data not shown).

After 30 min of reperfusion, the blood was collected and used to evaluate the role of R1 in the expression of adhesion molecules CD18 and CD11b of mouse peripheral neutrophils. As illustrated in Figure 9B, 30-minute reperfusion significantly enhanced the expression of both CD18 and CD11b compared to the control group. R1 treatment significantly inhibited the increment in the mean fluorescence intensity of CD18 induced by I/R, and also diminished, although not significantly, the increment in the fluorescence intensity of CD11b induced by I/R.

The influence of R1 on the concentration of enzymes in mouse peripheral blood was determined after 30 and 60 min of reperfusion (Figure 10). Thirty-minute or 60-minute reperfusion obviously increased the concentration of LDH, ALT and AST. However, treatment with R1 could not significantly blunt these increases, except for the activity of AST after 60-min reperfusion. I/R significantly increased the concentrations of IL-6 and MCP-1, but not TNF-α compared to the control group. R1 did not influence the increase in the concentration of IL-6 and MCP-1 induced by I/R (data not shown).

DISCUSSION

Terminal portal venules and central veins are two major types of vessels consisting of hepatic microvasculature, in addition to sinusoids. Using intravital microscopy, the present study demonstrated that the mouse SMA I/R induced a variety of disorders in hepatic microcirculation,

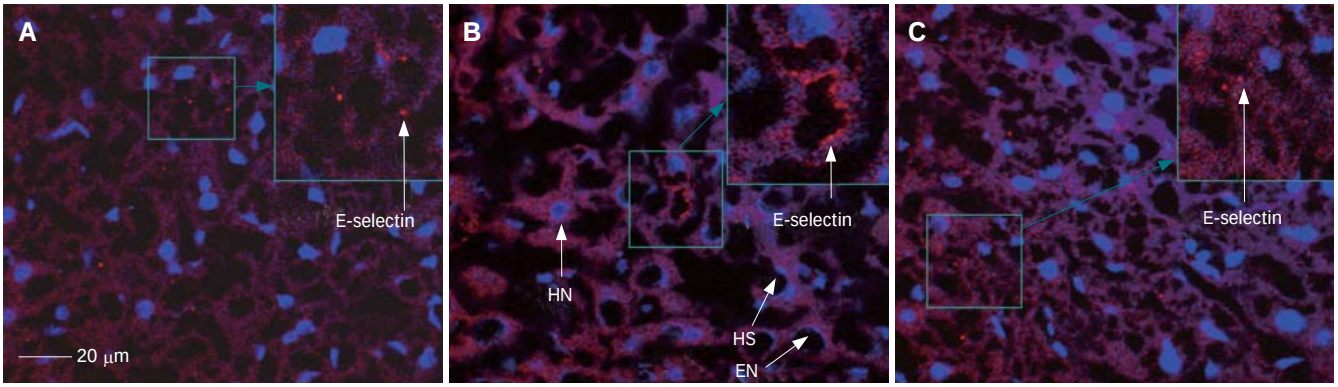


Figure 8 Expression of E-selectin in mouse hepatic sinusoid in sham (**A**), I/R (**B**) and R1 + I/R (**C**) after 30 min of reperfusion. HN: hepatocyte nucleus; EN: endothelium nucleus; HS: hepatic sinusoid; Bar indicates 20 μm.

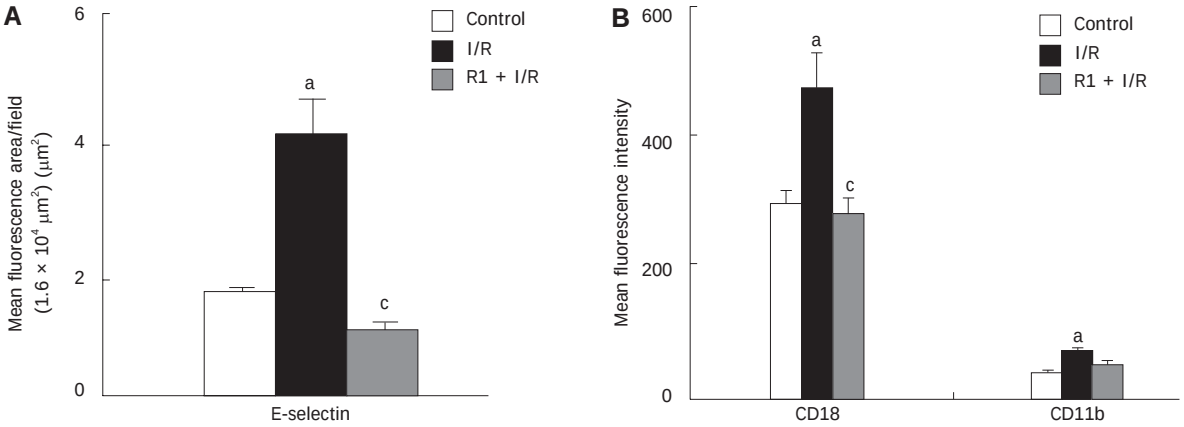


Figure 9 Effect of R1 on the expression of E-selectin in hepatic vessels (**A**) and CD18 and CD11b in neutrophils (**B**) of mice subjected to SMA I/R. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

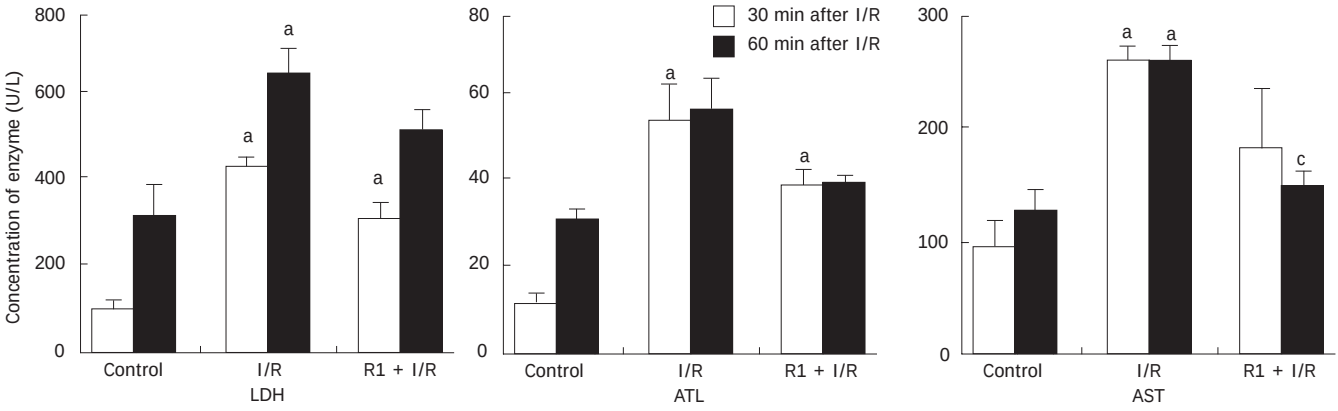


Figure 10 Effect of R1 on the concentration of LDH, ALT and AST in serum of mice subjected to SMA I/R. The results are presented as mean ± SE from 6 animals. $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$ *vs* I/R.

including the decreased diameters of terminal portal venules and central veins, RBC velocity in venules and the number of perfused sinusoids. Besides, leukocyte rolling and adhesion in hepatic venules and sinusoids were also promoted by the SMA I/R challenge. These results are in agreement with previous findings[2,4,24-26,30]. In the current study, treatment with R1 could remarkably attenuate hepatic microcirculatory disturbances in mice evoked by SMA I/R. PNS improved gut I/R-induced hepatic microcirculation disturbances and CP inhibited the adhesion of leukocytes to the hepatic vascular endothelium in rats induced by gut I/R, suggesting that R1 is at least one of the components of PNS that are responsible for its beneficial effect on hepatic microcirculation[19,24].

The concentration of ET-1 increases in serum and hepatic parenchyma in response to I/R[36], while NO is depleted by combination with I/R-evoked $^{-}O^{\cdot-}$[37], which concurs to bring about unbalance between ET-1

and NO, resulting in contraction of hepatic vessels. The present study revealed that administration of R1 attenuated I/R-elicited contraction of hepatic terminal portal venules and central veins that profoundly improved microcirculation in mice, suggesting that R1 may exert its action on the production of ET-1 and NO in the situation of I/R, which merits further study.

I/R-induced microcirculatory dysfunction and subsequent tissue injury are a complicated process consisting of multiple reactions, among which leukocyte recruitment is a crucial step mediated by the expression of a group of adhesion molecules on neutrophils and endothelial cells. It was reported that peroxide produced by SMA I/R degrades I-κB for activation of NF-κB and induces the expression of vascular endothelial E-selectin and ICAM-1[6-10], and also transfers L-selectin and adhesion molecules CD11b and CD18 from leukocyte cytoplasm to the cell surface[11-13,38], which initiates leukocyte rolling and adhesion ultimately. The ability of R1 to attenuate gut I/R-induced leukocyte rolling and adhesion in hepatic venules found in the present study is most probably due to its inhibiting effect on the expression of adhesion molecules on both leukocytes and endothelial cells, as suggested by the fact that pre-treatment with R1 could significantly blunt SMA I/R-induced expression of E-selectin on endothelium and CD18 on neutrophils.

It has been demonstrated that SMA I/R provokes a surge of peroxide release which activates NF-κB[6,7], leading not only to the expression of adhesion molecules on leukocytes and endothelium and subsequent leukocyte rolling and adhesion, but also to the explosive release of cytokines from leukocytes, endothelial and Kupffer cells[39-41], including TNF-α, IL-6 and MCP-1. The present experiment revealed that mouse gut I/R significantly increased the concentration of IL-6 and MCP-1, but not TNF-α in plasma. The reason is so far unknown. Moreover, no study is available to date concerning the antioxidant effect of R1 in I/R-induced hepatic microcirculatory disturbance, and it merits clarification if taking into account the fact that R1 depresses I/R-induced expression of adhesion molecules, but does not interfere with the increased concentrations of IL-6 and MCP-1 induced by I/R.

As expected, results of the present study show that the activities of ALT, AST and LDH of peripheral blood could slightly increased in response to 60-min SMA I/R, indicating that intestine I/R-induced microcirculatory disturbance in liver leads to injury of hepatic cells and dysfunction of the liver. Treatment with R1 significantly suppressed the activity of AST 60 min after reperfusion, suggesting that R1 can protect against hepatocyte injury induced by I/R. This beneficial effect is most likely related to its improving effect on hepatic microcirculatory disturbance, as mentioned above.

In summary, treatment with R1 considerably attenuates SMA I/R-induced hepatic microcirculatory disturbances, including decreased venular diameters, RBC velocity, the number of perfused sinusoids, as well as the increased leukocyte rolling and adhesion. R1 ameliorates SMA I/R-induced increase in the leukocyte rolling and adhesion in hepatic venules by inhibiting the expression of adhesion molecules on endothelial cells and neutrophils. We propose that it is the improving effect of R1 on microcirculatory disturbance that underlines its protecting function against I/R-induced hepatic injury.

COMMENTS

Background

Major abdomen surgery or organ transplantation initiates gut ischemia and reperfusion (I/R) leading to hepatic microcirculatory disturbance and subsequent liver injury, a manifestation that is closely correlated to the outcome of operation or transplantation and the living quality of patients as well. Thus, attenuating hepatic microcirculatory disturbance and liver injury elicited by gut I/R is of pivotal significance in clinic. Notoginsenoside R1 (R1), one of the saponins derived from *Panax notoginseng*, is reported to attenuate endotoxin-induced mesenteric microcirculatory disturbance in rats by inhibiting oxygen peroxide production and expression of adhesion molecules CD11b/CD18. It has been shown that R1 containing compound Chinese medicine preparation (cardiotonic pills) is able to ameliorate hepatic microcirculatory disturbance and liver injury elicited by gut I/R. R1 containing Chinese medicines is extensively applied in treatment of microcirculatory disturbance related diseases in China. However, no report is available regarding its attenuating effect on hepatic microcirculatory disturbance and liver injury induced by gut I/R.

Research frontiers

In present study, an animal model of hepatic microcirculatory disturbance was established by ligation of the superior mesenteric artery (SMA) in C57/BL mice for 15 min followed by 30-min reperfusion. The dynamics of vascular diameter, RBC velocity, rolling and adherent leukocytes was investigated in the hepatic terminal portal venule and central vein regions under inverted intravital microscope assisted by a 3CCD color camera and high speed video camera as well as a laser confocal microscope. Thirty minutes after reperfusion, the expression of adhesion molecules CD11b and CD18 on leukocytes in peripheral blood was estimated, and the expression of E-selectin and ICAM-1 in hepatic tissue was determined by immunohistochemistry. Sixty minutes after reperfusion, the levels of LDH, ALT, AST in peripheral blood were measured to explore the possible protective effect of R1 on hepatic microcirculatory disturbance and liver injury elicited by gut I/R.

Innovations and breakthroughs

By using a visualized microcirculatory research, results of the present study provide evidence for the first time that a pulse prior administration of R1 is able to prevent hepatic microcirculatory disturbance in mice induced by intestine I/R, including ameliorating contraction of hepatic terminal portal venule and central vein, resuming reduced velocity of RBCs, and inhibiting leukocyte rolling and adhesion in hepatic terminal portal venule and central vein, the later may be correlated to the inhibition of the expression of adhesion molecules and E-selectin on vascular endothelial cells and CD18 on leukocytes. R1 treatment could suppress AST level in peripheral blood, suggesting that R1 is able to prevent intestine I/R-induced hepatic microcirculatory disturbance and subsequent liver injury.

Applications

The findings in the present work support the utilization of R1 as a remedy in clinic to prevent hepatic microcirculatory disturbance and liver injury following major abdomen surgery or transplantation.

Peer review

This is a very interesting paper examining the effects of notoginsenoside R1 (R1) on hepatic microvascular function after gut I/R. The data are largely convincing. R1 has both vasodilatory and anti-inflammatory functions.

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CLINICAL RESEARCH

Increased susceptibility for intrahepatic cholestasis of pregnancy and contraceptive-induced cholestasis in carriers of the 1331T>C polymorphism in the bile salt export pump

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Abstract

AIM: To study the association of three common *ABCB11* and *ABCC2* polymorphisms (*ABCB11*: 1331T>C → V444A; *ABCC2*: 3563T>A → V1188E and 4544G>A → C1515Y) with intrahepatic cholestasis of pregnancy (ICP) and contraceptive-induced cholestasis (CIC).

METHODS: *ABCB11* and *ABCC2* genotyping data were available from four CIC patients and from 42 and 33 ICP patients, respectively. Allele-frequencies of the studied polymorphisms were compared with those in healthy pregnant controls and Caucasian individuals. Furthermore, serum bile acid levels were correlated with the presence or absence of the 1331 C allele.

RESULTS: The *ABCB11* 1331T>C polymorphism was significantly more frequent in cholestatic patients than in pregnant controls: C allele 76.2% (CI, 58.0-94.4) *vs* 51.3% (CI 35.8-66.7), respectively (P = 0.0007); and CC allele 57.1% (CI 36.0-78.3) *vs* 20% (CI 7.6-32.4), respectively (P = 0.0065). All four CIC patients were homozygous carriers of the C allele. In contrast, none of the studied *ABCC2* polymorphism was overrepresented in ICP or CIC patients. Higher serum bile acid levels were found in carriers of the 1331CC genotype compared to carriers of the TT genotype.

CONCLUSION: Our data support a role for the *ABCB11* 1331T>C polymorphism as a susceptibility factor for the development of estrogen-induced cholestasis, whereas no such association was found for *ABCC2*. Serum bile acid and γ-glutamyl transferase levels might help to distinguish *ABCB4*- and *ABCB11*-related forms of ICP and CIC.

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Key words: Cholestasis of pregnancy; Contraceptive-induced cholestasis; Bile salt export pump; Multidrug resistance associated protein 2; Pharmacogenetics

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INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP) and oral contraceptive-induced cholestasis (CIC) are two acquired forms of cholestasis, which are observed in otherwise healthy young women with a normal medical history. Both syndromes are rapidly reversible upon discontinuation of the hormonal challenge, which suggests that female sex hormones play a key pathogenic role in these forms of cholestasis[1,2]. In line with these observations, ICP usually occurs during the third trimester of pregnancy, when serum concentrations of estrogens and progesterone reach their peak[3,4]. Furthermore, women with ICP and female family members of ICP patients have an increased susceptibility to develop intrahepatic cholestasis under oral contraception[5].

A genetic predisposition for both types of hormonal cholestasis has been suspected based upon the strong regional clustering[6], the higher prevalence in female family members of patients with ICP[5,7] and the co-incidence with hereditary cases of progressive familial intrahepatic cholestasis[5,7]. Recently, mutations in the *ABCB4* gene that encodes the canalicular phospholipid flippase multidrug resistance protein

3 (MDR3) have been implicated in the development of ICP and CIC in a subset of affected patients[5,8-12]. MDR3-associated cases of hormonal cholestasis are associated with elevated serum γ-glutamyl transferase (γ-GT) levels in 80% of affected patients, which reflects cholangiocytic damage characteristic of MDR3 dysfunction[11].

In the same study, the majority of ICP women without *ABCB4* mutations exhibited normal γ-GT levels[11], which suggests a different pathogenic mechanism in this subset of patients. Dysfunction of the bile salt export pump (BSEP) or the multidrug resistance associated protein 2 (MRP2) have, therefore, been proposed as alternative candidate proteins involved in the pathogenesis of hormonal cholestasis. BSEP constitutes the predominant bile salt efflux system of hepatocytes, which mediates the cellular excretion of numerous conjugated bile salts into the bile canaliculus[13-16]. In contrast, the bilirubin transporter MRP2 is the main driving force for bile-salt-independent bile flow through canalicular excretion of reduced glutathione[17,18]. Given their important roles in bile formation and bilirubin secretion, inherited and acquired dysfunction of these proteins can lead to severe cholestatic syndromes and conjugated hyperbilirubinemia, respectively[19-21].

In hormonal cholestasis, *in vitro* inhibition of BSEP by estrogen and progesterone metabolites has been proposed as an underlying pathophysiological mechanism[22]. BSEP inhibition by estrogen and progesterone metabolites takes place from the luminal side of the bile canaliculus (so-called trans inhibition), which requires previous MRP2-mediated canalicular secretion of conjugated metabolites[22,23]. Therefore, MRP2 dysfunction might contribute to this form of cholestasis. While sequencing of *ABCB11* in unrelated ICP women has not revealed the presence of disease-causing BSEP mutations[11], only little attention has so far been paid to the possible pathogenic role of functional *ABCB11* and *ABCC2* polymorphisms. Recent observations have suggested that a non-synonymous polymorphism in exon 13 of the *ABCB11* gene (1331T>C) is overrepresented in drug-induced cholestatic liver injury[24]. The same polymorphism has recently been observed more frequently in ICP women compared to healthy controls, pointing towards a possible role of this polymorphism as a susceptibility factor for ICP and CIC[25]. Furthermore, two non-synonymous *ABCC2* polymorphisms (V1188E and C1515Y) showed significant differences in hepatic MRP2 expression levels compared to the wildtype sequence, which could be relevant for the extent of BSEP trans inhibition[25].

The aim of the present study was, therefore, threefold: (1) to compare allele frequencies of the aforementioned *ABCB11* and *ABCC2* polymorphisms in a prospectively recruited group of patients with ICP and CIC; (2) to define the relative risk of the different polymorphisms for the development of ICP; and (3) to determine the extent of the increase in serum bile acid levels as marker of cholestasis in the presence of the different *ABCB11* 1331T>C genotypes.

MATERIALS AND METHODS

Patients and controls

After approval by the Ethics Committee of the University Hospital of Zurich and written informed consent from all participating individuals, blood samples for DNA extraction were obtained from Caucasian patients with ICP or CIC. The total population of analyzed individuals consisted of two different groups: 25 patients (21 ICP_{new} patients and four CIC patients) were prospectively recruited for this study, and a second group of 20 patients (ICP_{old}) had already been described in a previous study by Pauli-Magnus and coworkers[11].

Two hundred and five Caucasian volunteers and patients without cholestasis, as well as Caucasian women with uneventful pregnancies (n = 40), served as a control population for BSEP (*ABCB11*) and MDR3 (*ABCB4*) genetic variants. These controls have already been described in previous studies[11,25,26]. Specifically, pregnant controls were all healthy, as defined by normal serum levels of transaminases, bilirubin, γ-GT, alkaline phosphatase (AP) and bile acids. Caucasian controls from the study of Pauli-Magnus and coworkers[26] (n = 95) were healthy volunteers recruited for participation in phase I studies, with uneventful medical history and normal blood biochemistry. Neither of these two control groups took any regular medication. In the case of the Caucasian control population of Meier and coworkers[25] (n = 110), most patients suffered from extrahepatic malignancies, and cholestatic disease was excluded in all patients. Furthermore, none of these patients used medication known to be associated with the development of cholestasis.

For lack of DNA availability, only 110 out of 205 Caucasian controls could be used for MRP2 sequencing. For the same reason, a new group of Caucasian women with uneventful pregnancies (n = 42) had to be collected for the MRP2 variants. Demographic data and pregnancy course of these women did not differ from the previous control group.

Diagnosis of ICP was based upon: (1) a clinical history of pruritus, which occurred in the third trimester of pregnancy; (2) the presence of laboratory abnormalities suggestive of ICP: fasting serum bile acid $\geqslant$ 1.5 ULN (upper limit of normal) and/or serum AP levels $\geqslant$ 1.5 ULN and/or alanine aminotransferase (ALT) levels $\geqslant$ 1.5 ULN; and (3) spontaneous resolution of clinical symptoms and laboratory findings after delivery. Diagnosis of CIC was based upon laboratory abnormalities as defined for ICP and the exclusion of preexisting liver disease defined by: (1) a negative serology for hepatitis A, B and C; (2) the exclusion of other preexisting medical conditions that could explain liver injury, such as congestive heart failure, systemic infection, or malignancy; (3) normal liver ultrasound; and (4) a clear causal relationship to drug intake. Each case of ICP and CIC was evaluated by at least one obstetrician and one hepatologist, as well as by a clinical pharmacologist.

Full length *ABCB4* and *ABCB11* sequencing data were already available from the control groups, as well as from ICP_{old} patients. To allow detection of additional *ABCB4* and *ABCB11* mutations in the new group, complete sequencing of these two genes was also performed in the 25 newly recruited patients. Genotyping of *ABCC2* included all CIC patients, as well as 17 out of 21 patients from the ICP_{new} group and 16 of 21 patients in the ICP_{old} group, which yielded a total number of 33 patients for *ABCC2*

Table 1 Primers and probes of real-time PCR for allelic discrimination of *ABCC2* SNPs in Caucasians

cDNA position[1]	SNP	Exon	Amino acid change	Eense-/antisense primer	Probes[2]
1249	G>A	10	V417I	5'-CCAACTTGGCCAGGAAGGA-3'/	VIC 5'-CTGTTTCTCCAACGGTGTA-3'
				5'-GGCATCCACAGACATCAGGTT-3'	FAM 5'-ACTGTTTCTCCAATGGTGTA-3'
3563	T>A	25	V1188E	5'-GCACCAGCAGCGATTTCTG-3'/	VIC 5'-ACACAATGAGGTGAGGAT-3'
				5'-AGGTGATCCAGGAAAAGACACATTT-3'	FAM 5'-ACAATGAGGAGAGGAT-3'
4544	G>A	32	C1515Y	5'-GTAATGGTCCTAGACAACGGGAAG-3'/	VIC 5'-AGAGTGCGGCAGCC-3'
				5'-CCAGGGATTTGTAGCAGTTCTTCAG-3'	FAM 5'-ATTATAGAGTACGGCAGCC-3'

[1]cDNA sequence from GenBank accession numbers NM_000392 starting at the ATG; [2]For each SNP two probes were designed and labeled with the fluorescent reporter dyes VIC (allele 1) and FAM (allele 2). SNP: Single nucleotide polymorphism.

genotyping. In nine patients (four ICP_{new} and five ICP_{old}), no *ABCC2* genotyping could be performed for lack of DNA availability.

Sequencing and genotyping

Isolation of DNA and DNA sequencing was done at Epidauros Biotechnology AG, Bernried, Germany. Genomic and cDNA sequences were derived from known sequences (*ABCB4*: AC005068.2 for non-coding exons -3 to 1 and coding exons 2 and 3; AC006154.1 for exons 4 to 12; AC0005045.2 for exons 13 to 28; and NM_000443.2 for cDNA; *ABCB11*: GenBank accession number AC008177.3 for promoter and exons 1 to 21; AC069165.2 for exons 22 to 28 and NM_003742.2 for cDNA).

ABCB4 and ABCB11: Sequencing of *ABCB4* covered a proximately 8000 bp, including (1) 2000 bp of the upstream promoter region and non-coding exon -3 to 1 and, (2) coding exons 2-28 and 100-350 bp of the intronic sequence around each exon. For *ABCB11*, sequencing covered 10000 bp including (1) non-coding exon 1 and 2400 bp of the upstream promoter region and, (2) coding exons 2-28 and 100-350 bp of the intronic sequence around each exon. Primers for genomic DNA were designed to span all exons and at least 100 bp of the flanking intronic sequence at the 5' and 3' end of each exon. The DNA sequence of purified PCR fragments was analyzed on an ABI3700 capillary sequencer (ABI, Weiterstadt, Germany) and assembled using the phredPhrap, Consed and PolyPhred software (University of Washington). Details regarding the primers, optimized PCR conditions and subsequent purification and sequencing of the fragments are available at info@epidauros.com.

ABCC2: Three non-synonymous polymorphisms with a potential impact on MRP2 function and expression were chosen for genotyping[25]: 1249G>A variant (V417I, rs2273697), 3563T>A (V1188E, rs17222723) and 4544G>A (C1515Y, rs8187710). Genotyping was performed with the Custom TaqMan SNP Genotyping Assays procedure (Applied Biosystems, Foster City, CA, USA) which contained a sense- and an antisense primer and two probes, labeled with fluorescent reporter dyes, either VIC or 6-Fam at the 5' end and a non-fluorescent quencher at the 3' end to distinguish between alleles 1 and 2, respectively. Primer and probe sequences for individual SNPs are given in Table 1. Probe solution (0.625 μL) and 12.5 μL of 2 × Universal PCR Master Mix (Applied Biosystems) were brought to 25 μL with 20 ng of genomic DNA. PCR reaction (2 min at 50℃, followed by 10 min at 95℃ and 40 cycles of 15 s at 92℃ and 1 min at 60℃). Allelic discrimination was processed with an ABI PRISM 7700 Sequence Detector.

Statistical analysis

Genotype distribution, allelic frequencies and odds ratios (ORs) are given with 95% CI. In *ABCB11*, formal statistical analysis was only performed for the 1331T>C polymorphisms (rs2287622), whereas for *ABCC2* analysis, it included two highly linked polymorphisms. No correction according to Bonferroni was, therefore, required. Differences investigated in our study apply to a proportion of diseased *versus* non-diseased individuals within the whole population, using an unmatched case control design. Response (ICP *versus* non-ICP) and predictors (T *versus* C) were both binary variables and were therefore best condensed into a 2 × 2 table. Differences in genotype distribution between patients and controls were calculated with the χ^2 test, and difference in allelic frequencies between two groups was performed using a 2 × 2 Fisher exact test. $P \leqslant 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

A total of 25 unrelated patients with estrogen-associated intrahepatic cholestasis were prospectively enrolled in this study, 21 with ICP and four with oral CIC. Demographic data and laboratory findings in ICP patients are given in Table 2. Only two patients showed elevated γ-GT levels > 1.5 ULN, while total bile acid levels were elevated in all patients in whom it was determined (16 out of 21; range, 1.7-17.3 ULN). Three patients had a previous history of ICP; three pregnancies were twin pregnancies, and one patient experienced cholestasis under previous oral contraception.

Characteristics of patients with CIC are given in Table 3. One patient showed elevated γ-GT levels. Total bile acid levels were elevated in all three patients in whom it was determined (three out of four; range, 1.6-22.3 ULN). Oral contraceptive preparations used in the four patients contained comparable amounts of ethinylestradiol (20-35 μg) while the progesterone-like portion ranged from 50 to 150 μg. All patients had a liver biopsy done for strictly diagnostic reasons, which showed intrahepatic cholestasis in three patients. One patient had a previous history of ICP.

Table 2 New group of patients with ICP (ICP$_{new}$)

Patient ID	Age (yr)	Liver parameters					Comments		Genotypes of SNPs		
		ALT (ULN)	AP (ULN)	γ-GT (ULN)	tBili (ULN)	tBA (ULN)	No of preg/ No ICP	Others	*ABCB11* 1331T>C (V444A)	*ABCC2* 3600T>A (V1188E)	*ABCC2* 4581G>A (C1515Y)
1	36	0.9	2.3	3.3	0.8	10.7	2/1		CC	TA	GA
2	31	1.6	2.5.	0.3	0.8	17.3	3/2		TC	TA	GA
3	35	8.9	2.3	1	0.5	10	1/1		TC	TT	GG
4	29	1.2	3	0.6	0.5	7	2/1		CC	TT	GG
5	42	11.6	1.4	0.4	0.8	6	1/1		TC	TT	GG
6	28	4.8	1.8	0.2	0.8	4.7	2/1	Twins	CC	TA	GA
7	32	11.9	3	1.2	0.9	3.6	nd		CC	TT	GG
8	16	5.2	2	0.4	nd	3.3	nd		TC	TT	GG
9	38	6.2	1.2	1.1	0.4	3	2/1		TC	TT	GG
10	23	1.6	1.3	1.3	0.8	2.4	1/1	Twins	TC	TT	GG
11	30	0.4	0.9	0.2	0.5	2.4	3/1		TC	TT	GG
12	22	0.3	1.8	0.1	0.5	2.1	nd		TC	TA	GA
13	32	0.2	0.9	0.7	0.2	2	2/1		TT	TT	GG
14	30	0.8	1.2	0.3	0.6	1.7	1/1		CC	TT	GG
15	28	7.5	2.3	0.9	0.6	nd	nd		TC	TT	GG
16	31	0.9	2.1	1.2	0.5	8.1	1/1		CC	TT	GG
17	20	3.4	1.1	0.4	0.5	2.6	1/1		TT	TT	GG
18	24	7.2	1.3	0.4	0.8	nd	1/1		CC	nd	nd
19	31	8.9	1.3	1.2	1.5	nd	2/2		CC	nd	nd
20	32	11	2.2	0.4	3.1	nd	3/3	Pruritus with contraceptives	CC	nd	nd
21	41	10.9	2.9	5.7	1.2	nd	1/1	Twins	CC	nd	nd
Summary median (Q1; Q3)	31 (28; 32)	4.8 (0.9; 8.9)	1.8 (1.2; 2.3)	0.6 (0.4; 1.2)	0.6 (0.5; 0.8)	3.5 (2.4; 7.3)					

n/a: Not available; ALT: Alanine aminotransferase; tBili: Total bilirubin; tBA: Total bile acids.

Table 3 Characteristics of patients with oral CIC

Patient ID	Oral contraceptive	Age (yr)	Exposure time	Liver parameters					Comments		Genotypes of SNPs		
				ALT (ULN)	AP (ULN)	γGT (ULN)	tBili (ULN)	tBA (ULN)	Clinical features	Histology	*ABCB11* 1331T>C (V444A)	*ABCC2* 3600T>A (V1188E)	*ABCC2* 4581G>A (C1515Y)
1[1]	30 μg ethinylestradiol/ 75 μg gestodene	32	nd	4.9	1.7	1	10.9	22.3	Jaundice	Intrahepatic cholestasis	CC	TT	GG
2	30 μg ethinylestradiol/ 150 μg levonorgestrel	15	21 d	1	3	1	4.2	nd	Jaundice, nausea, pruritus	Extensive intrahepatic cholestasis	CC	TT	GG
3	35 μg ethinylestradiol/ 50 μg levonorgestrel	40	2 yr	3.9	2.8	3.6	0.5	1.6	Pruritus	Bland	CC	TT	GG
4	35 μg ethinylestradio/ 2 mg cyproteron	34	nd	1	1.3	nd	2.8	1.6	Jaundice	Extensive canalicular cholestasis, mild portal inflamation	CC	TA	GA

[1]Patient exhibited previous episodes of ICP.

Sequence analysis

ABCB4 and ABCB11: Sequence analysis in the 25 newly recruited patients with estrogen-associated cholestasis revealed no disease-associated non-synonymous mutations in *ABCB4* or *ABCB11*. Furthermore, in line with previous findings[11], no *ABCB4* polymorphism was found to be overrepresented in the ICP and CIC groups compared to pregnant women without cholestasis and healthy Caucasian individuals. All of the detected genetic variants in *ABCB11* and *ABCB4* were in Hardy Weinberg equilibrium.

In contrast, the *ABCB11* 1331T>C → V444A polymorphism was significantly more frequent in ICP and CIC patients compared to the two control groups. Specifically, the CC genotype was encountered in 57.1% of all ICP patients (ICP$_{new}$, 47.6% and ICP$_{old}$, 67.7%) and 100% of CIC patients compared to 20 and 32.2% in pregnant

Table 4 Genotype distribution of non-synonymous *ABCB11* variant site 1331T>C in patients and controls

Genotype SNP	ICP_{old}		ICP_{new}		ICP_{total}		Pregnant controls		Caucasian controls	
	n (%)	95 % CI	*n* (%)	95 % CI	*n* (%)	95 % CI	*n* (%)	95 % CI	*n* (%)	95 % CI
ABCB11 1331T>C (V444A)	21 (100)		21 (100)		42 (100)		40 (100)		205 (100)	
TT (VV)	-	-	2 (9.5)	0.0-22.1	2 (4.8)	0.0-13.9	7 (17.5)	5.7-29.3	38(18.5)	13.2-23.9
CC (AA)	14 (67.7)	46.5 -86.8	10 (47.6)	26.3-69.0	24 (57.1)	36.0-78.3	8 (20)	7.6-32.4	66 (32.2)	25.8-38.6
TC (VA)	7 (33.3)	13.2-53.5	9 (42.9)	21.7-64.0	16 (38.1)	17.3-58.9	25 (62.5)	47.5-77.5	101 (49.3)	42.4-56.1
Frequency C allele	35 (83.3)	67.4-99.3	29 (69.0)	49.3-88.8	64 (76.2)	58.0-94.4	41 (51.3)	35.8-66.7	233 (56.8)	50.1-63.6
Frequency T allele	7 (16.7)	0.7-32.6	13 (31.0)	11.2-50.7	20 (23.8)	5.6-42.0	39 (48.8)	33.3-64.2	177 (43.2)	36.4-50.0
ABCC2 3563T>A (V1188E)	16 (100)		17 (100)		33 (100)		42 (100)		110 (100)	
TT (VV)	15 (93.8)	71.3-98.6	13 (76.5)	52.3-90.4	28 (84.8)	68.9-93.3	37 (88.1)	74.3-96.1	95 (86.4)	68.9-93.3
AA (EE)	-	-	-	-	-	-	-	-	1 (0.9)	0.0-5.0
TA (VE)	1 (3.1)	0.0-15.8	4 (23.5)	9.6-47.7	5 (15.2)	6.7-31.1	5 (11.9)	3.9-25.7	14 (12.7)	7.1-20.5
ABCC2 4544G>A (C1515Y)	16 (100)		17 (100)		33 (100)		42 (100)		110 (100)	
GG (CC)	15 (93.8)	71.3-98.6	13 (76.5)	52.3-90.4	28 (84.8)	68.9-93.3	36 (85.7)	71.4-94.6	95 (86.4)	68.9-93.3
AA (YY)	-	-	-	-	-	-	-	-	1 (0.9)	0.0-5.0
GA (CY)	1 (3.1)	0.0-15.8	4 (23.5)	9.6-47.7	5 (15.2)	6.7-31.1	6 (14.3)	5.4-28.6	14 (12.7)	7.1-20.5

Results are given with 95 percent confidence interval (95% CI).

Table 5 1331T>C (V444A): Fisher's exact test and ORs for the presence of homozygous CC variant and the C allele in the different groups

	CC *vs* TT			C *vs* T		
	Fisher	Odds ratio	95% CI	Fisher	Odds ratio	95% CI
IC_{Pold} *vs* Pregnant controls	0.0041	nd[1]	-	0.0004	4.8	2.2-15.0
IC_{Pold} *vs* Caucasian controls	0.0029	nd[1]	-	0.0005	3.8	1.9-11.1
IC_{Pnew} *vs* Pregnant controls	0.1082	4.4	0.7-27.2	0.0441	2.1	1.0-4.7
IC_{new} *vs* Caucasian controls	0.1461	2.9	0.6-13.8	0.0850	1.7	0.9-3.4
IC_{Ptotal} *vs* Pregnant controls	0.0065	10.5	1.9-63.7	0.0007	3.0	1.7-6.4
IC_{Ptotal} *vs* Caucasian controls	0.0025	6.9	1.6-32.1	0.0006	2.4	1.5-4.5

[1]Could not be determined, as TT = 0.

women without cholestasis and healthy Caucasian controls, respectively (Table 4). In line with these findings, the ORs of C *versus* T were 3.0 (1.7-6.4) for all ICP patients (ICP_{new} + ICP_{old}) *versus* healthy pregnant control women (ICP_{new}, 2.1; 1.0-4.7 and ICP_{old} 4.8; 2.2-15.0) (Table 5 and Figure 1). With the exception of this polymorphism and two intronic variants that were found to be closely linked to the 1331T>C polymorphism in previous studies [intron 13: (+70) C>T and intron 14 (+32) T>C][26], the allele frequency of the remaining common variants in the patients with ICP and CIC was comparable to that observed in healthy pregnant and Caucasian controls. Due to the small sample size, no significance levels could be calculated for the CIC group. However, all patients in this group were homozygous for the C at position 1331, which is highly suggestive of an overrepresentation of this allele compared to the control groups.

ABCC2: The 1249G>A polymorphism was not found in our patients. In contrast, 3563T>A and 4544G>A were strongly linked and distributed similarly in all groups (Table 4). No significant difference in the frequency of these two polymorphisms was observed between affected ICP and CIC patients and healthy controls. Heterozygous carriers of the 3563A and the 4544A alleles were found in 15.2% of ICP patients (ICP_{new}, 23.5% and ICP_{old}, 3.1%) compared to 11.9 and 12.7% in pregnant women without cholestasis and healthy Caucasian controls, respectively (Table 4). Furthermore, one CIC patient was a heterozygous carrier for the two variant alleles at positions 3563 and 4544.

Relation of serum bile acid levels and the ABCB11 1331T>C genotype

For correlation of bile acid levels with the corresponding genotype at position 1331 of *ABCB11*, ICP and CIC samples were analyzed together. Bile acid levels were available for 16 out of 21 ICP_{new} patients, seven out of 20 ICP_{old} patients, and three out of four CIC patients, which yielded a total of 26 samples (CC, 14 patients; CT, 10 patients; and TT, two patients). Interindividual variability in serum bile acid levels was high and ranged from 1.7 to 22.3 ULN and 1.7 to 17.3 ULN in CC and CT patients, respectively. Serum bile acid levels gradually increased from carriers of the TT genotype to carriers of the CC genotype, with medians of 2.3 ULN (Q1, 2.2; Q3, 2.5), 3.2 ULN (Q1, 2.4; Q3, 6.2) and 4.4 ULN (Q1, 2.2; Q3, 7.8) for TT, TC and CC, respectively (Figure 2).

DISCUSSION

We investigated the risk association between different *ABCB11* and *ABCC2* polymorphisms in ICP and CIC, and correlated different genotypes with serum bile acid levels

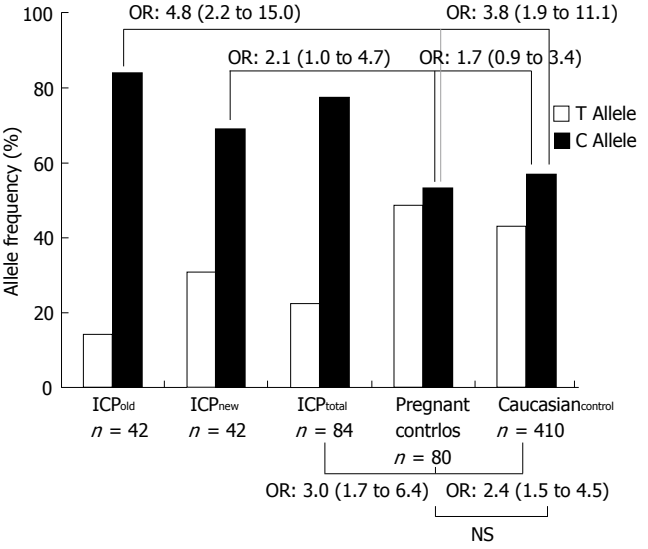


Figure 1 Allelic frequency of the T allele (white panel) and C allele (black panel) of the ABCB11 1331T>C (1331T>C) polymorphism. 21 ICP_{new} patients (42 alleles); 21 ICP_{old} patients (42 alleles); 42 ICP_{total} patients (84 alleles); 20 $ICP_{control}$ patients (40 alleles); 205 $Caucasian_{control}$ patients (410 alleles). Allelic frequencies and ORs are given with 95% CI. Groups were compared with Fisher's exact test.

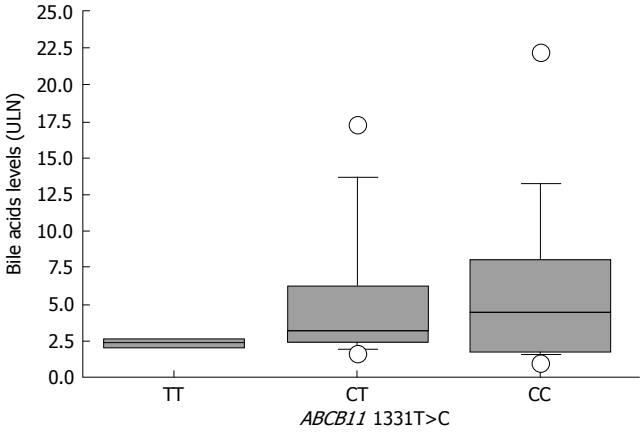


Figure 2 Bile acid levels in patients harboring different 1331C>T genotypes. TT: two patients; CT: 10 patients; CC: 14 patients.

as a marker of cholestasis. In our group of 25 patients with estrogen-associated cholestasis (21 with ICP and four with CIC), there was a highly significant association between the presence of the C allele at position 1331 of *ABCB11* and the presence of cholestasis, which confirms preliminary results from another collective of ICP women, in whom such an overrepresentation was first observed[11]. While *in vitro* function of both BSEP variants, as measured by taurocholate transport activity, is comparable[24], BSEP expression in healthy liver tissue of Caucasian individuals has recently been found to be lower in carriers of the 1331C allele[25]. Such differences in hepatic BSEP expression levels might offer one valuable explanation for the increased susceptibility to the development of cholestasis under specific circumstances, such as hormonal challenges. Furthermore, serum bile acids as a marker of *in vivo* BSEP function was influenced by the underlying genotype. Lowest bile acid levels were observed in patients with the TT genotype and highest levels in carriers of the CC genotype. Although the accepted level of statistical significance was not reached due to high interindividual variability, this observation is in line with the hypothesis that the underlying genotype at position 1331 is a determinant of BSEP function, and hence contributes to the individual risk of developing cholestasis.

The homozygous state for the 1331T>C polymorphism has only recently been observed in a very severe case of pregnancy-associated cholestasis with serum bile acid levels > 40-fold above the ULN. Interestingly, decreased BSEP expression levels were found in a liver biopsy obtained from this patient[27]. Although this patient carried an additional *ABCB4* mutation, the presence of decreased hepatic BSEP expression and highly elevated bile acid levels strongly support a BSEP-related mechanism as a predominant pathogenetic factor. The same patient also developed severe cholestasis under previous use of oral contraceptives, which supports the notion that the same polymorphism also predisposes to oral CIC.

In our group, seven patients (from ICP_{old}) carried additional *ABCB4* mutations, while no such mutations were detected in the remaining 35 out of 42 ICP patients (ICP_{old}, 14 and ICP_{new}, 21). This finding suggests that the *ABCB11* 1331T>C polymorphism independently contributes to an individual's risk for developing cholestasis under certain conditions. On the other hand, it can be speculated whether the combination of the 1331T>C polymorphism with *ABCB4* mutations might be a risk constellation for a severe disease course, as observed by Keitel and coworkers[27].

In contrast, no association was found between the presence of the non-synonymous polymorphisms at positions 1188 and 1515 of MRP2 and the presence of ICP or CIC. A possible pathogenic role of these two polymorphisms in ICP and CIC was suspected based upon the genotype-dependent alteration in hepatic MRP2 expression levels in healthy human liver tissue[25]. Specifically, heterozygous carriers of the glutaminic acid at position 1188 and tyrosine at position 1551 showed significantly higher levels of MRP2 in their liver than homozygous carriers of valine and cysteine, respectively[25]. As BSEP inhibition by estrogen and progesterone metabolites requires prior MRP2-mediated secretion into the bile canaliculus, high MRP2 expression was suspected as a risk factor for the development of estrogen-dependent cholestasis[22].

Several conclusions can be drawn from this study. First, our data point toward a pathogenic relevance of the *ABCB11* 1331T>C polymorphism in ICP and CIC. While these types of cholestasis are so far mainly attributed to different disease-causing mutations in *ABCB4*[5,11,12], our data support a clear association between the presence of a frequent *ABCB11* polymorphism and ICP. Interestingly, all of the patients with CIC were homozygous carriers of the C allele at position 1331. It can be speculated that lower estrogen levels in CIC compared to second or third trimester pregnancy require two low-function alleles to result in cholestasis. Furthermore, the 1331T>C variant was also found to be associated with other inherited and acquired forms of cholestasis, such as benign recurrent intrahepatic cholestasis and drug-induced cholestasis[24,28,29]. This suggests a role for this polymorphism as a risk factor for different cholestatic conditions, which have so far been regarded as different disease entities[20,30].

Second, while γ-GT levels are elevated in ICP patients who carried a disease-causing *ABCB4* mutation[11], serum bile acid levels are influenced by the BSEP genotype at position 444 of *ABCB11*. It can, therefore, be speculated that these two parameters allow us to clinically distinguish between MDR3- and BSEP-related forms of estrogen-related cholestasis, as it is already done for progressive forms of inherited familial intrahepatic cholestasis[5,21]. From a prognostic point of view, this might help to distinguish patients that carry a common susceptibility factor from those who carry a disease-causing *ABCB4* mutation, which in some cases, has been associated with disease progression[7,11,12,31]. Third, although a pathogenic involvement of MRP2 in estrogen-induced cholestasis has longly been suspected, common *ABCC2* polymorphisms have not been associated with the development of cholestasis. We did not exclude the presence of disease-associated *ABCC2* mutations in our group, but normal bilirubin levels in all but one patient suggests no major MRP2 dysfunction, which should result in a Dubin Johnson phenotype[32].

In summary, our data support a role for the *ABCB11* 1331T>C polymorphism as a susceptibility factor for the development of estrogen-induced cholestasis, whereas no such association was found for *ABCC2*. Serum bile acid and γ-GT levels might help to distinguish *ABCB4* and *ABCB11*-related forms of ICP and CIC.

COMMENTS

Background

Intrahepatic cholestasis of pregnancy (ICP) and oral contraceptive-induced cholestasis (CIC) are two acquired forms of cholestasis, which are observed in otherwise healthy young women with a normal medical history. The bile salt export pump (BSEP, *ABCB11*) and the multidrug resistance protein 2 (MRP2, *ABCC2*) might be of pathogenetic importance in both conditions.

Research frontiers

A genetic predisposition for both types of hormonal cholestasis has been suspected based upon the strong regional clustering, the higher prevalence in female family members of patients with ICP, and the co-incidence with hereditary cases of progressive familial intrahepatic cholestasis. While mutations in the *ABCB4* gene that encodes the canalicular phospholipid flippase multidrug resistance protein 3 (MDR3) have been implicated in the development of ICP and CIC in a subset of affected patients, the role of genetic variants in *ABCB11* and *ABCC2* remains unclear.

Innovations and breakthroughs

Our data support a role of the *ABCB11* 1331T>C polymorphism as a susceptibility factor for the development of estrogen-induced cholestasis, whereas no such association was found for *ABCC2*. Serum bile acid and γ-GT levels might help to distinguish *ABCB4*- and *ABCB11*-related forms of ICP and CIC.

Applications

While the clinical consequences of such findings are still uncertain at this time, they provide important new insights in the role of genetically determined differences in canalicular transporter expression and function for the development of estrogen-induced cholestasis. In the future, the integration of different factors that predict cholestasis might be used to counsel pregnant patients or to avoid certain medications in susceptible patients.

Terminology

ICP: Intrahepatic cholestasis of pregnancy; CIC: contraceptive-induced cholestasis; BSEP: Bile Salt Export Pump (*ABCB11*); MRP2: Multidrug Resistance Protein 2 (*ABCC2*); MDR3: Multidrug Resistance Protein 3 (*ABCB4*).

Peer review

The study characterized a potential underlying defect in the subgroup of normal γ-GT ICP patients and contributes to a clinical risk assessment for the future. This study from a group with longstanding experience in transporter genomics is well designed and presented in a clearly written manuscript.

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CLINICAL RESEARCH

Factors that impact health-related quality of life in adults with celiac disease: A multicenter study

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Abstract

AIM: To evaluate the factors involved in the impairment of health-related quality of life (HRQOL) in patients with celiac disease.

METHODS: A multicenter, cross-sectional prospective study was performed in patients with celiac disease who completed two HRQOL questionnaires: the gastrointestinal quality of life index (GIQLI) and the EuroQol-5D (EQ).

RESULTS: Three hundred and forty patients (163 controlled with a gluten-free diet, and 177 newly diagnosed with a normal diet) were included. The GIQLI score was significantly better in patients on a gluten-free diet (GFD) than in non-treated patients on their usual diet, both in terms of the overall score (3.3 *vs* 2.7, respectively; $P < 0.001$), as well as on the individual questionnaire dimensions. Both the preference value of the EQ as the visual analogue scale were significantly better in treated than in non-treated patients (0.93 *vs* 0.72 $P < 0.001$ and 80 *vs* 70 $P < 0.001$, respectively). Variables significantly associated with a worse HRQOL score were female gender, failure to adhere to a GFD, and symptomatic status.

CONCLUSION: In untreated celiac disease, the most important factors that influence patient perception of health are the presence of symptoms and a normal diet. HRQOL improves to levels similar to those described in the general population in celiac disease patients well controlled with a GFD.

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Key words: Celiac disease; Health status; Quality of life; Gluten-free diet

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INTRODUCTION

Celiac disease (CD) is a chronic immuno-inflammatory enteropathy that appears in genetically predisposed patients. Patients with this disease develop characteristic small-intestinal-mucosal changes, due to hypersensitivity to gluten. Several epidemiological factors, including a dramatic increase in the diagnosis of CD[1] and changes in its presentation, with the appearance of atypical forms, that may even mimic functional diseases, as well as the fact that it affects people of any age[2] mean that CD is currently considered a common and important health-care problem. It affects approximately 1%-2% of adults in Western European populations[3].

Although the understanding of the immunology and physiopathology of CD is extensive[4,5], the impact of the disease from the patient's point of view has received less attention. The chronic nature of the condition, together with the limitations imposed by the need to follow a permanent restrictive diet, substantial numbers of physician visits, the risk of associated diseases and potential complications, mean that CD can have a considerable negative impact on health-related quality of life (HRQOL). Due to the lack of disease-specific instruments to measure HRQOL in CD patients, generic questionnaires, such as the SF-36 or EuroQol-5D (EQ), have been used. Overall perception of HRQOL is rated

as bad or fair by 63% of patients before diagnosis of CD, and improves to 77% after treatment[6]. However, at least one study has indicated that treated CD patients rate their overall well-being, as measured with a visual analog scale (VAS), quite highly, with 83.6% of patients being rated as 'very well' or 'well'[7]. Apart from dietary treatment, other factors have been suggested to influence the HRQOL in CD patients. Some disease-related factors, such as the presence of symptoms or associated co-morbidity, as well as the type of diagnosis used (symptom-detected *vs* screening-detected), have also been shown to impair CD patients' HRQOL[8,9]. Non-disease-related factors, such as female gender, also have a negative impact on HRQOL[10], although this is also true in other chronic diseases[11]. The impairment of HRQOL in untreated celiac disease and its improvement after treatment with a gluten-free diet (GFD), has been confirmed using generic multidimensional HRQOL measures[12]. However, there is no agreement as to whether the HRQOL of treated CD patients is similar[8,13,14] or not[15,16] to that of the general population.

The aims of the present study were to measure and compare the HRQOL in treated and untreated CD patients using condition-specific and generic measures of HRQOL, and to analyze the factors impacting on HRQOL in these patients. A further objective was to compare scores on the generic measure of HRQOL for the two study groups with general population reference scores for the same instrument (EQ). To this end, we performed a multicenter study in a representative sample of patients from different parts of Spain, including both large and small cities, and both island and mainland regions.

MATERIALS AND METHODS

Study subjects

Subjects were adult outpatients with CD who attended the Digestive Services Units of seven different Spanish hospitals, over a one-year period. Diagnosis was based on current serological and histological criteria[17]. A total of 340 CD patients were included. At the time of inclusion, patients were stratified into two groups, according to whether they were already following a GFD (GFD group) or whether they had not yet started on the GFD (pretreatment group).

Procedure

All patients completed a questionnaire on demographic details, current symptoms, information on the disease, and issues related to the GFD. They also completed the Gastrointestinal Quality of Life Index (GIQLI) questionnaire and the EQ, two generic HRQOL instruments.

Patient compliance following the GFD was measured using an adapted version of the self-administered questionnaire developed by Morisky *et al*[18]. The questionnaire consisted of four items which measure the degree of treatment compliance, answered using dichotomous response options (yes/no). Two questions asked about unintentional lack of compliance ("sometimes I forget my diet/sometimes I do not comply carefully with my diet"), while the other two questions dealt with intentional lack of compliance ("when I feel well I sometimes discontinue my diet/when not feeling well I sometimes discontinue my diet"). If either question 3 or 4 was answered affirmatively, the patient was considered to have voluntarily discontinued his or her diet. If either question 1 or 2 was answered in the affirmative, the patient was considered to have involuntarily neglected or forgotten his or her diet. This questionnaire was originally developed to measure compliance with medication, and was adapted for use in the current population by substituting drugs with GFD. In a previous study[2], many patients suggested that they never forgot about their diet; for that reason, a fifth option was added to the scale ("I never forget about my diet"). Patients checking this answer were considered good compliers.

In an ancillary study, 17 patients from the pre-treatment group completed the questionnaires both at inclusion, when they had not yet started on the GFD, and at least 6 mo following treatment with GFD.

HRQOL assessment

HRQOL was assessed using two generic questionnaires. GIQLI is a self-administered questionnaire designed to assess HRQOL in patients with gastrointestinal diseases. It was chosen because it has been translated and validated for use in Spain[19]. The GIQLI consists of 36 items grouped into five domains of health (gastrointestinal symptoms, physical dysfunction, social dysfunction, emotional dysfunction and treatment effects). Responses are scored on a 4-point Likert scale, in which 4 corresponds to the highest level of functioning. The instrument produces five dimension scores and an overall score ranging from 0 to 4, with a higher score reflecting better HRQOL.

EQ is a short self-administered generic utility measure that provides both a descriptive profile and an overall index for HRQOL. The EQ includes five dimensions (mobility, personal care, daily activities, pain and anxiety-depression) answered on a 3-point scale ranging from no problems (level 1) to extreme problems (level 3). Combining one level of severity from each of the five dimensions generates a number of discrete health states that can be assigned preference values ranging from 0, which represents worst health status, to 1 (best health status). The EQ also includes a VAS that ranges from 0 (worst imaginable health status) to 100 (best imaginable health status), on which patients were asked to mark the point that best reflected their health status on the day of the interview. The EQ has also been translated and validated in Spanish[20]. As a reference, normal values for a representative sample of 12245 members of the Spanish general population also exist for the EQ, and we used these as a control for the patients included in the present study[21,22].

Statistical analysis

The Kolmogorov-Smirnov test showed that most of the study variables did not have a normal distribution. Descriptive analyses of socio-demographic, clinical and HRQOL data were therefore performed using medians and 25th and 75th percentiles. Qualitative variables were described as proportions. Comparisons between variables were performed using the Mann-Whitney *U*, Kruskal-Wallis or Fisher tests as appropriate. In the pre-treatment group, HRQOL was also analyzed depending on whether the diagnosis was performed on the basis of symptoms or on positive serology.

Table 1 Socio-demographic and clinical characteristics of patients *n* (%)

	Pre-treatment	GFD
Number	177	163
Age (yr)	44 (30-50)	37 (28-47)[a]
Sex (male/female)	74/103	42/121
Smoking status		
Smoker	29 (16)	33 (25)
Non-smoker	148 (84)	130 (75)
City of residence		
< 100 000 inhabitants	69 (43)	61 (39)
100 000-500 000	72 (45)	65 (41)
> 500 000	18 (12)	31 (20)
Family status		
Single	57 (33)	67 (42)
Married	108 (63)	84 (53)
Widowed	5 (4)	8 (5)
Educational level		
No studies	7 (4)	4 (2)
Primary	56 (33)	38 (24)
Secondary	55 (32)	61 (38)
University studies	52 (31)	55 (36)
Occupational status		
Employee/self-employed	67 (41)	94 (62)
Retired/pensioner	36 (22)	14 (9)
Housewife	30 (18)	16 (10)
Student	12 (7)	19 (12)
Unemployed	17 (12)	10 (7)
Disease duration (mo)	-	48 (24-84)
Duration of symptoms before diagnosis (mo)	24 (7-84)	12 (5-40)
Presence of symptoms at inclusion	118 (80)	52 (33)[b]
Presence of associated diseases at inclusion	42 (41)	41 (37)

Results are expressed as medians and (25th and 75th percentiles) or absolute values and (percentage). [a]$P < 0.05$, [b]$P < 0.01$.

Three multiple linear regression models were developed to identify independent variables that influence HRQOL. Dependent variables in the three models were the overall GIQLI score, the EQ preference value index, and the EQ VAS scores. Independent variables in all models were GFD, sex, age, duration of symptoms, and the binary variables were existence of symptoms and presence of CD-associated diseases. Results obtained for the multiple linear regression model were the regression coefficient, *t*-test statistics, *P* values, and the variance inflation factor. The *t* test established whether the independent variables contributed to predicting the dependent variables. Higher *t*-test values indicate that the independent variable more strongly predicts the dependent variable. It has been considered that each independent variable contributes to predicting the dependent variable when $P < 0.05$. The variance inflation factor, a measure of multicollinearity, measures the inflation of the standard error of each regression coefficient for an independent variable due to redundant information in other independent variables. If the variance inflation factor is 1.0, there is no redundant information in the other independent variables. The level of statistical significance for the multiple regression model was set at $P < 0.05$.

RESULTS

Patient and disease characteristics

A total of 340 patients from seven hospitals in different areas of Spain were included. Table 1 summarizes the socio-demographic and clinical characteristics, according to whether they were in the GFD group ($n = 163$) or the pre-treatment group ($n = 177$).

There were no relevant differences in demographic characteristics between the two groups. Age ranged from 15 to 78 years. Treated patients were younger than non-treated patients [median age 37 (28-47) *vs* 44 (30-50) years, respectively; $P < 0.05$], but the difference was not considered clinically relevant. The sample characteristics were also in accordance with the results of previous epidemiological studies of CD in Spain[2], with patients being young adults and predominantly female and non-smokers. Most patients lived in small to medium-sized cities, with < 500 000 inhabitants.

The duration of disease since diagnosis was not evaluated in the normal diet group because they were included at the time of diagnosis. There were no statistically significant differences in duration of symptoms before diagnosis of CD between treated and pre-treated patients. The disease presented in the classic form in 47 (32%) patients in the GFD group and in 13 (11%) patients in the normal diet group ($P < 0.01$). The most frequently presenting non-classical forms of CD included anemia (32%), cutaneous lesions (9%), and hypertransaminasemia (6%). Approximately 40% of patients had associated diseases, such as thyroid diseases (7%), selective IgA deficiency (3%), type I diabetes mellitus (5%), depression (11.1%), and chronic inflammatory arthropathy (8%). The percentage of asymptomatic patients at inclusion was significantly higher in the treated GFD group than in the normal diet group (80% *vs* 33%, $P < 0.01$). In the GFD group, 117 patients (73%) were classified as good compliers on the basis of their Morisky scores, 36 (22%) reported that they occasionally involuntarily neglected or forgot the diet, and eight (5%) had voluntarily discontinued the diet. Again in the GFD group, 47% of patients reported complete disappearance of symptoms since being on the diet, and 43% reported a significant improvement in symptoms. Eight per cent of patients reported a small improvement in symptoms, and 2% considered that their symptoms had not changed with treatment.

Description of HRQOL in CD

Figure 1 shows the overall score and the five dimensions of GIQLI for the GFD and pre-treatment groups. Patients in the GFD group reported significantly ($P < 0.001$) better HRQOL than patients in the pre-treatment group for the overall score and the gastrointestinal symptoms, and in the physical, social and emotional dysfunction dimensions.

Patients in the pre-treatment normal diet group scored best on GIQLI treatment effects domain [4.0 (3.0-4.0)], while the domains with the worst scores were physical dysfunction [2.3 (1.4-2.9)], emotional dysfunction [2.4 (1.8-3.0)], and gastrointestinal symptoms [2.8 (2.4-3.3)]. These results suggested that most impaired domains of health in newly diagnosed CD patients were related to symptomatic and emotional dimensions. In GFD patients, the best scored domain was also the treatment domain [4.0 (3.0-4.0)] and the worst scored was emotional dysfunction [3.0 (2.4-3.4)], which

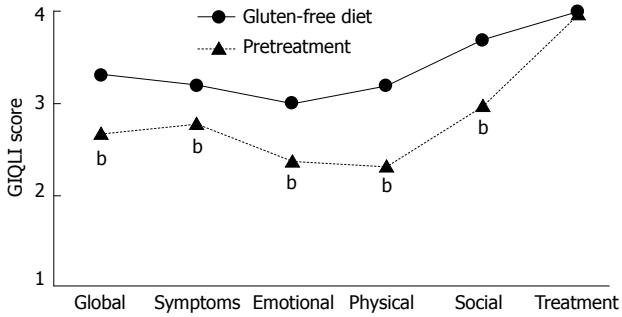


Figure 1 Median global and dimension scores of GIQLI for GFD (circles) and untreated (triangles) patients. Scores were significantly higher ($P < 0.001$) for all dimensions except treatment. [b]$P < 0.001$.

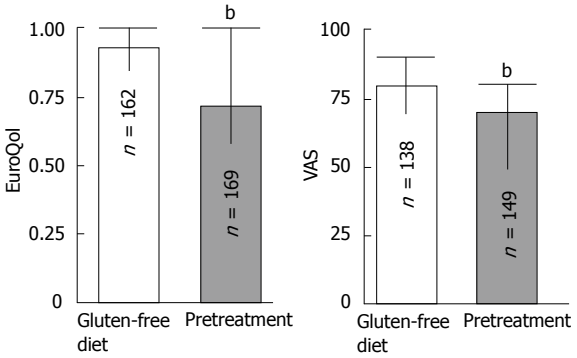


Figure 2 Median scores and 25th and 75th percentiles for the tariff and VAS of the EQ for GFD (white columns) and normal diet (grey columns). Scores were significantly better ($P < 0.001$) for patients in the GFD group. [b]$P < 0.001$.

suggested that, although improved by treatment, the most sustained impairment affected the emotional dimension.

Median scores on the EQ preference value index and VAS are shown in Figure 2. The median EQ preference value was significantly higher in GFD patients than in the normal diet group [0.93 (0.85-1.0) *vs* 0.72 (0.58-1.0) respectively, $P < 0.001$]. The median VAS score was also significantly higher in GFD patients than in the normal diet group [80 (70-90) *vs* 70 (50-80) respectively, $P < 0.001$]. Presence (levels of severity 2 and 3) or absence (level 1) of problems for each EQ dimension for the GFD and normal diet patients are shown in Table 2. At diagnosis, the normal diet group patients reported problems with significantly greater frequently than those in the GFD group on all five dimensions of the EQ, which confirmed that GFD improved patient perception of health. In the normal diet group, problems were reported most frequently in the pain/discomfort (62%) and anxiety/depression (54.5%) dimensions, and least frequently in the self-care dimension (10.2%). In the GFD group, problems were reported most frequently on the EQ anxiety/depression dimension (33.9%), in consonance with the low score of the emotional dimension in the GIQLI observed in this group of patients.

Factors influencing HRQOL in CD

Table 3 shows the results of the multiple regression analysis, which took the GIQLI overall score as a dependent variable for all patients. The analysis suggested

Table 2 Description of presence or absence of problems for each EQ dimension according to treatment *n* (%)

Variable	Pre-treatment		Gluten-free diet	
	No problems	Some problems	No problems	Some problems
Mobility	130 (74.3)	45 (25.7)	146[b] (90.1)	16 (9.9)
Self-care	158 (89.8)	18 (10.2)	161[b] (99.3)	1 (0.7)
Usual activities	113 (64.5)	62 (35.5)	135[b] (83.3)	27 (16.7)
Pain/Discomfort	67 (38)	109 (62)	117[b] (72.8)	45 (27.7)
Anxiety/Depression	80 (45.5)	96 (54.5)	107[b] (66.1)	55 (33.9)

[b]$P < 0.001$.

Table 3 Results of the multiple regression modeling with global GIQLI score as a dependent factor

Independent variable	*t*-test statistics	*P* value	Variance inflation factor
Pretreatment/treatment	4.989	< 0.001	1.31
Age	-0.725	0.469	1.31
Gender (m/f)	-2.684	0.008	1.06
Smoking habit	1.038	0.301	1.02
Symptoms duration	-1.813	0.072	1.08
Presence of symptoms	-4.932	< 0.001	1.31
Associated diseases	-1.824	0.070	1.18

Table 4 Results of the multiple regression modeling with the preference value of the EQ score as the dependent factor

Independent variable	*t*-test statistics	*P* value	Variance inflation factor
Pretreatment/treatment	3.20	0.002	1.31
Age	0.44	0.663	1.20
Gender (m/f)	-1.12	0.261	1.07
Smoking habit	0.58	0.559	1.02
Symptoms duration	-1.61	0.109	1.08
Presence of symptoms	-5.09	< 0.001	1.31
Associated diseases	-2.61	0.01	1.18

that the presence of symptoms, normal diet and female gender were the strongest determinants ($P < 0.001$) for a worse perception of HRQOL.

Similar results were obtained using the linear regression analysis with the EQ preference value index (Table 4) and the EQ VAS (Table 5) as the dependent variables, and included the same independent variables as described earlier. Presence of symptoms and normal diet represented significant determinants of worse HRQOL. In this case, the presence of CD-associated diseases was also associated with poorer HRQOL.

In the pre-treated normal diet group, results of the comparison of patients diagnosed according to the presence of symptoms or a positive serology ($n = 26$) are shown in Figure 3. HRQOL was significantly worse in patients diagnosed through symptoms rather than through serologic tests without observable symptoms. The worst HRQOL was, therefore, found in women who had been recently diagnosed due to the presence of symptoms, who were in the normal diet group, and who had associated diseases.

In the GFD group, specific potential factors that may be related to HRQOL, such as compliance or response to

Table 5 Results of the multiple regression modeling with the VAS of the EQ score as a dependent factor

Independent variable	*t*-test statistics	*P* value	Variance inflation factor
Pretreatment/treatment	2.97	0.003	1.37
Age	0.81	0.420	1.23
Gender (m/f)	-1.48	0.141	1.11
Smoking habit	-0.49	0.624	1.02
Symptoms duration	-2.71	0.007	1.08
Presence of symptoms	-5.99	< 0.001	1.40
Associated diseases	-2.50	0.013	1.17

diet, were assessed. There were no statistically significant differences in median overall GIQLI score between patients classified as good compliers (n = 117), and those who involuntarily neglected or forgot their diet (n = 36), and those who had voluntarily interrupted their diet (n = 8) [median GIQLI (IQR) scores, 3.3 (2.8-3.5), 3.2 (2.8-3.4), and 2.9 (2.6-3.3), respectively]. In contrast, GIQLI scores were significantly higher (better HRQOL) in the 73 patients who reported achieving complete control of symptoms with the GFD, than in patients reporting a partial or non-response (n = 84) [3.4 (3.1-3.6)] *vs* [3.0 (2.7-3.4), $P < 0.001$]. With reference to the potential effect of GFD duration on HRQOL, the duration of treatment and GIQLI overall score were slightly correlated ($r = 0.19$, $P < 0.05$) according to the Spearman rank correlation test. Similar correlation was observed with the EQ preference value index and the EQ VAS ($r = 0.20$, $P < 0.05$ and $r = 0.15$, P = NS).

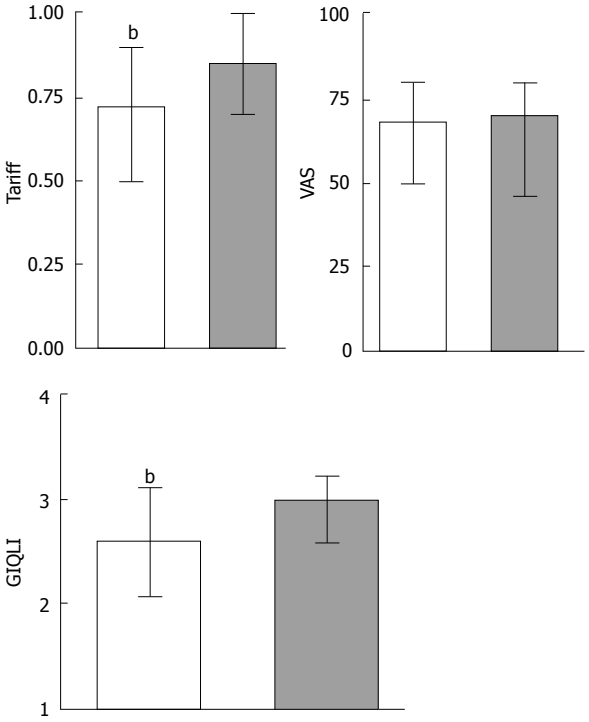


Figure 3 Median scores and 25th and 75th percentiles for the global GIQLI (left panel) and EQ index and VAS (right panel) in the pre-treated normal diet CD patients (white columns represents patients diagnosed due to symptoms and grey columns those diagnosed by serological tests). Scores were significantly worse ($P < 0.05$) for patients diagnosed due to symptoms compared to those with isolated serologic tests. [b]$P < 0.001$.

Intensity of impairment of HRQOL in CD

Although statistical comparisons between CD patients' EQ scores and those described for the Spanish general population (median preference value of 1.0 and VAS score of 80) were not performed, it seems apparent that treated patients do not have different preference values and VAS scores to the Spanish general population. In contrast, preferences and VAS scores of the normal diet group were worse than those of the general population. According to the distribution of EQ VAS scores in the Spanish general population (21), CD patients perceive their health as good to excellent (VAS scores between 70 and 100), while untreated patients with newly diagnosed CD have VAS scores corresponding to fair health.

Changes in HRQOL of CD before and after treatment

Seventeen newly diagnosed patients [11 women, six men, median age 48 (34-60) years] completed the HRQOL questionnaire before initiating treatment and after 6-23 mo with GFD. Paired comparisons using the Wilcoxon signed rank test showed that treatment improved significantly the GIQLI overall score [from a median of 2.6 (2.3-3.0) to 2.9 (2.7-3.4), P <0.001] and the VAS score [from 70 (55-70) to 77 (70-87) respectively, $P < 0.01$]. However, the median EQ preference value increase after treatment did not reach statistical significance [0.85 (0.69-1.0) to 0.87 (0.71-1.0), P = NS].

DISCUSSION

To analyze how CD affects HRQOL and to determine which factors are associated, we administered two generic instruments to measure HRQOL in two groups of CD patients: 163 patients following a GFD and 177 newly diagnosed patients who were on a normal diet. A potential limitation of the study is a selection bias for patients because we only included hospital-controlled patients. Results on the GIQLI and EQ questionnaires suggest that the HRQOL of untreated recently diagnosed CD patients is significantly impaired, on almost all of the dimensions on both instruments and on the overall score. It is not surprising then that these patients' assessment of their overall health on the EQ should be equivalent to a rating of only fair health. Patients before starting a GFD only scored well on the treatment effects dimension on the GIQLI questionnaire. Although these patients have not initiated the GFD, the highly-scored dimension was treatment, probably because this item refers to "feeling let down by treatment effects" and most patients have received prior diet or drug recommendations, or because patients tend to respond neutrally to items in this domain. By contrast, CD patients controlled with a GFD reported significantly better HRQOL, and in fact had EQ scores that were very similar to those of a representative sample of the Spanish general population[21,22]. These results study are in line with those reported very recently by the Canadian Celiac Association[23]. In that study, it was found that SF-12 summary scores for CD patients were similar to the normative Canadian data, except for females and newly diagnosed patients.

The importance of adequate compliance with a GFD is confirmed by the multivariate analysis, in which it was shown that GFD exerted a major influence on HRQOL,

whether using the condition-specific GIQLI or the generic EQ instrument. To avoid the problem of between-person variability in the determination of the effect of GFD on HRQOL, in a subgroup of newly diagnosed patients, the HRQOL was re-measured after at least 6 mo on GFD. Results of that ancillary study confirmed that treatment of CD improves HRQOL. The multivariate analysis also indicated that the presence of symptoms and comorbidity were other independent variables that significantly influenced HRQOL in CD patients. These results suggest that compliance with GFD and adequate control of symptoms are decisive, in terms that CD patients may recover to satisfactory levels of self-perceived health. The duration of improvements in HRQOL in CD patients following a GFD could not be deduced from our study, but it has been suggested that such improvements may be maintained for as long as 20 years[14].

Although our study confirmed that a GFD improves symptoms, with 90% of patients reporting a clinically significant improvement, as well as raising CD patients' levels of HRQOL to those reported by the general population, other studies, such as that by the Westchester Celiac Sprue Support Group have observed negative effects of a GFD on HRQOL in areas such as dining out, travel and family life[24]. These aspects were not covered in depth by either of the HRQOL instruments included here, and they should be considered for inclusion in future studies.

Advances in the serologic diagnosis of CD mean that the disease is recognized at increasingly early times, even in asymptomatic patients. Presumably as a result of the absence of symptoms in patients diagnosed by serologic screening, as opposed to patients diagnosed by clinical symptoms, it has been suggested that screening-detected CD patients have better HRQOL than those diagnosed based on symptoms[10,25]. There is no agreement as to whether a GFD improves HRQOL independently of whether diagnosis was based on serologic screening or on symptoms, with some evidence suggesting that improvements in HRQOL occur in both types of patients[25], and other evidence pointing out that they are only found in symptom-detected patients[10]. Our study also provides some evidence that symptom-detected patients have significantly lower overall scores on the GIQLI and on the EQ preference index than serologic-detected patients, which suggests that the former have better self-perceived health on diagnosis. However, the nature of the present study meant that it was not possible to determine whether diet-induced improvements in HRQOL were related or not to the type of diagnosis.

The only non-disease-related variable that had a significant influence on HRQOL was gender, with women scoring significantly poorer on the GIQLI questionnaire. It has been suggested that in treated CD patients, low scoring of SF-36 is confined to female patients[15]. However, in our study, the influence of gender on HRQOL was less clear because, although female sex was a significant independent variable for poorer perception of HRQOL as measured by the GIQLI, the effect was not seen on either the EQ preference value index or on the EQ VAS.

Our results suggest that untreated newly diagnosed CD has a significant negative impact on several domains of HRQOL, and that HRQOL is more impaired in symptomatic patients and in those with CD-associated diseases. On the other hand, HRQOL improves to levels observed in the general population when CD is controlled with a GFD. From our observations, it can be concluded that the assessment of HRQOL in CD patients is relevant because it improves physicians' knowledge of the implications of the disease, and helps patients to recognize the general impact of the disease. Additionally, the fact that patients following a GFD report similar levels of HRQOL as members of the general population should encourage patients to adhere to the GFD.

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COMMENTS

Background

Celiac disease (CD) is a chronic immuno-inflammatory enteropathy that appears in genetically predisposed patients, who develop characteristic small-intestinal-mucosal changes, due to hypersensitivity to gluten. The chronic nature of the condition, together with the limitations imposed by the need to follow a permanent restrictive diet, substantial numbers of physician visits, the risk of associated diseases and potential complications, mean that CD can have a considerable negative impact on health-related quality of life (HRQOL). The impairment of HRQOL in untreated CD and its improvement after treatment with a gluten-free diet (GFD), has been suggested using generic multidimensional HRQOL measures.

Research frontiers

There is a lack of information relative to some areas in the research of HRQOL in CD. There is no agreement as to whether the HRQOL of treated CD patients reaches that of the general population, the factors involved in HRQOL impairment, and in the use of more specific instruments to measure the HRQOL of CD patients.

Innovations

Two instruments to measure HRQOL in two groups of CD patients: 163 patients following a GFD and 177 newly diagnosed patients who were on a normal diet containing gluten. Results on the gastrointestinal quality of life index GIQLI and EuroQol-5D EQ questionnaires suggested that the HRQOL of untreated, recently diagnosed CD patients was significantly impaired, on almost all of the dimensions on both instruments and on the overall score. Patients, before starting a GFD, considered their overall health equivalent to a rating of only fair. CD patients controlled with a GFD reported significantly better HRQOL, and in fact had EQ scores that were very similar to those of a representative sample of the Spanish general population. The importance of diet on CD was confirmed in an ancillary study in which HRQOL was re-measured after at least 6 mo on GFD. Results of that ancillary study confirm that treatment of CD improves HRQOL. Our study also provided some evidence that symptom-detected patients had significantly lower overall scores on the GIQLI and on the EQ preference index than serologic-detected patients, which suggested that the former had better self-perceived health on diagnosis. To determine the factors involved in HRQOL impairment in CD, a multivariate analysis using the condition-specific GIQLI or the generic EQ instrument was performed. Results of that analysis suggested that the variables significantly associated with a worse HRQOL were female gender, failure to follow a GFD, and symptomatic status.

Applications

The results of the present study suggest that compliance with a GFD and adequate control of symptoms are decisive in terms that CD patients may recover to satisfactory levels of self-perceived health. The assessment of HRQOL in CD patients is relevant because it improves physicians' knowledge of the implications of the disease and helps patients to recognize the general impact of the disease. The fact that patients following a GFD reached similar levels of HRQOL as members of the general population should encourage patients to adhere to a GFD.

Terminology

HRQOL has a recognized importance to evaluate, manage and follow patients with chronic diseases, such as CD. Different types of instruments for measuring HRQOL have been introduced, with the most important being the questionnaires. There are generic questionnaires and disease-specific questionnaires. Although a specific questionnaire for CD has recently been published, the most widely used instruments to measure HRQOL in CD are the generic questionnaires. GIQLI is a self-administered questionnaire designed to assess HRQOL in patients with gastrointestinal diseases. The GIQLI consists of 36 items grouped into five domains of health (gastrointestinal symptoms, physical dysfunction, social dysfunction, emotional dysfunction and treatment effects). Responses are scored on a 4-point Likert scale, in which 4 corresponds to the highest level of functioning. EQ is a self-administered generic utility measure that provides both a descriptive profile and an overall index for HRQOL. The EQ includes five dimensions (mobility, personal care, daily activities, pain and anxiety–depression) answered on a 3-point scale ranging from no problems (level 1) to extreme problems (level 3). Combining one level of severity from each of the five dimensions generates a number of discrete health states that can be assigned preference values ranging from 0, which represents worst health status, to 1 (best health status). The EQ also includes a VAS that ranges from 0 (worst imaginable health status) to 100.

Peer Review

This is a well designed study addressing an important aspect of celiac disease which adds considerably to what is already known.

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CLINICAL RESEARCH

Clinical value of fecal calprotectin in determining disease activity of ulcerative colitis

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Abstract

AIM: To investigate possibility and clinical application of fecal calprotectin in determining disease activity of ulcerative colitis (UC).

METHODS: The enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of calprotectin in feces obtained from 66 patients with UC and 20 controls. C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), acid glycoprotein (AGP) were also measured and were compared with calprotectin in determining disease activity of UC. The disease activity of UC was also determined by the Sutherland criteria.

RESULTS: The fecal calprotectin concentration in the patients with active UC was significantly higher than that in the inactive UC and in the controls (402.16 ± 48.0 μg/g *vs* 35.93 ± 3.39 μg/g, 11.5 ± 3.42 μg/g, $P < 0.01$). The fecal calprotectin concentration in the inactive UC group was significantly higher than that in the control group ($P < 0.05$). A significant difference was also found in the patients with active UC of mild, moderate and severe degrees. The area under the curve of the receiver operating characteristics (AUC^{ROC}) was 0.975, 0.740, 0.692 and 0.737 for fecal calprotectin, CRP, ESR and AGP, respectively. There was a strong correlation between the fecal calprotectin concentration and the endoscopic gradings for UC ($r = 0.866$, $P < 0.001$).

CONCLUSION: Calprotectin in the patient's feces can reflect the disease activity of UC and can be used as a rational fecal marker for intestinal inflammation in clinical practice. This kind of marker is relatively precise, simple and noninvasive when compared with other commonly-used markers such as CRP, ESR and AGP.

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Key words: Fecal calprotectin; Disease activity; Ulcerative colitis; Enzyme-linked immunosorbent assay

INTRODUCTION

Ulcerative colitis (UC) is a chronic, idiopathic inflammatory bowel disease characterized by remission of disease activity. The incidence and the prevalence rates of UC are increasing in China[1]. It is important to accurately evaluate intestinal mucosa inflammation in the management of these patients, particularly for the assessment of therapeutic effectiveness. Colonoscopy and biopsy are useful in the assessment of intestinal mucosa inflammation of patients with UC, but these examinations can be a heavy burden to the patient[2,3]. Clinical evaluations including laboratory tests such as C-reactive protein (CRP)[4-6], erythrocyte sedimentation rate (ESR)[7], acid glycoprotein (AGP)[8], and platelet count[9,10], have been used for the determination of disease activity of UC, but none of them are specific for gut inflammation[11]. Therefore, a new marker that will be more sensitive and specific for determination of disease activity of UC is urgently needed in clinical practice.

An alternative approach to the assessment of the presence of intestinal inflammation[12] is to analyze the whole gut lavage fluid or to quantitate the protease resistant neutrophil derived proteins such as lactoferrin[13,14] in the patient's feces, and this approach can be non-invasive for the patient. Calprotectin is one of these proteins. It is a major protein in the neutrophilic granulocytes and the macrophages[15], which accounts for 60% of the total protein in the cytosol fraction in these cells[16,17]. This kind of protein can resist metabolic degradation caused by intestinal bacteria, and the protein is relatively stable in stools for up to one week at room temperature[18]. It can differentiate between patients with organic or non-organic intestinal disease, and can be useful in detecting colorectal cancer and inflammatory disorders, and can also be useful in predicting a relapse of inflammatory bowel disease[19].

Our study was aimed at the measurement of the concentration of calprotectin in the feces of the UC patient and at the comparison of it with commonly-used markers in clinical practice, such as CRP, ESR and AGP.

MATERIALS AND METHODS

Study subjects

Sixty-six patients with UC (age, 38.97 ± 2.39 years) were enrolled in the study, including 15 patients with proctitis, 22 with left-sided colitis, and 29 with pancolitis. Of the patients, 44 were hospitalized. The patients' disease activities were assessed according to the Sutherland criteria[20] in which a score of more than two was considered to indicate the active stage of the disease. The control group consisted of 20 subjects (age, 38.95 ± 3.59 years) with no confirmed abnormality in the upper or lower digestive tract.

Methods of stool collection

The patients were instructed to defecate directly into a polystyrene container. The stool samples were stored at -70℃ until the time of measurement.

Measurement of fecal calprotectin by ELISA

The stool samples were thawed, and 50-100 mg of the sample was suspended with 2500-5000 μL of the fecal extraction buffer, and was homogenized; then, the suspernatant was diluted to 1:50, and the calprotectin was analyzed by the enzyme-linked immunosorbent assay (ELISA) using the Nycotest Phical ELISA kit (Nycomed, Norway). Microcapture and immunoaffinity-purified rabbit anticalprotectin conjugated with alkaline phosphatase was used for the development. The ELISA read the absorbance at 405 nm for 96-well plates. The results of the sample tests were evaluated from the standard curves. CRP, ESR and AGP were measured in the clinical laboratory, West China Hospital, based on the instructions provided by the reagent manufacturer.

Statistical analysis

Statistical analysis was performed using the statistical package SPSS 11.5. The data were expressed as means ± SD. The Mann-Whitney test was used to assess differences in the laboratory parameters between the groups, and Spearmans' correlation was used to analyze the correlation between the parameters. All the P values were two tailed; P values < 0.05 were considered statistically significant. The receiver operating characteristics (ROC) (sensitivity and specificity) were assessed by the curve analysis as described by Henderson[21].

RESULTS

Concentrations of fecal calprotectin, CRP, ESR and AGP in patients with UC and in controls

There was a significant difference in the fecal calprotectin concentration between the patients with active UC and the patients with inactive UC ($P < 0.01$) (Table 1, Figure 1). The calprotectin concentration was significantly greater in the patients with inactive UC than in the controls ($P < 0.05$). The patients with active UC had higher levels of CRP, ESR and AGP than the patients with inactive UC and the controls ($P < 0.05$), but there was no significant difference between the patients with inactive UC and the controls.

Relationship between the concentrations of fecal calprotectin, CRP, ESR and AGP and the disease activity index (DAI) in UC

As shown in Figure 2, the concentrations of fecal calprotectin, CRP, ESR and AGP in UC had a good correlation with DAI. The correlation coefficients between DAI and the concentrations of fecal calprotectin, CRP, ESR and AGP were 0.866, 0.492, 0.433 and 0.533, respectively. This association was strongest for fecal calprotectin and weakest for ESR.

Specificity and sensitivity

The correspondence between the DAI-based classification of the active or the inactive disease status and the classification based on the parameter cut-offs was analyzed for each parameter (Table 2) and was expressed as the percentage of the samples that were correspondingly identified (specificity and sensitivity). Specificity was highest for fecal calprotectin, and lowest for AGP. The specificity rates for fecal calprotectin, CRP, ESR and AGP were 79.4%, 69.0%, 68.9% and 65.5%, respectively. The sensitivity for fecal calprotectin was relatively high, but was relatively low for CRP. The sensitivity rates for fecal calprotectin, CRP, ESR and AGP were 91.9%, 62.2%, 64.9%, and 67.6%, respectively. The ROC curves showed the trade-off between specificity and sensitivity for fecal calprotectin (the area under the curve, AUC, 0.975 ± 0.015; $P < 0.001$), for CRP (AUC, 0.740 ± 0.061; $P < 0.001$), for ESR (AUC, 0.692 ± 0.064; $P < 0.01$), and for AGP (AUC, 0.737 ± 0.062; $P < 0.001$) (Figure 3). The AUC^{ROC} of fecal calprotectin was greater than that of CRP, ESR or AGP ($P < 0.01$).

DISCUSSION

Chronic relapsing and remitting inflammation of the gastrointestinal tract is the hallmark of UC. One of the most prominent histological features observed in UC is infiltration of the neutrophils into the inflamed mucosa at an early stage of inflammation. The neutrophils are major sources of inflammatory cytokines, chemokines proteases, active lipids, and reactive oxygen derivatives, as well as a full complement of factors needed to exacerbate mucosal inflammation and tissue injury[22-25] The fecal calprotectin excretion of indium-labeled autologous granulocytes has for a long time been suggested as the gold standard test in assessing bowel inflammation in inflammatory bowel disease. However, as it involves an exposure to radiation and prolonged fecal collection, which is unpopular with patients and laboratory staff, it is only used as a research tool. In patients with inflammatory bowel disease, a three day excretion of indium-labeled granulocytes correlated well with a daily excretion and a one-off fecal calprotectin level[26].

In this study, we focused on the evaluation of any relationship that might exist between the mucosal neutrophil infiltration represented by calprotectin, CRP, ESR, AGP and the UC disease activity represented by the Sutherland criteria. The DAI score of UC is the sum score of the following four parameters (each scoring

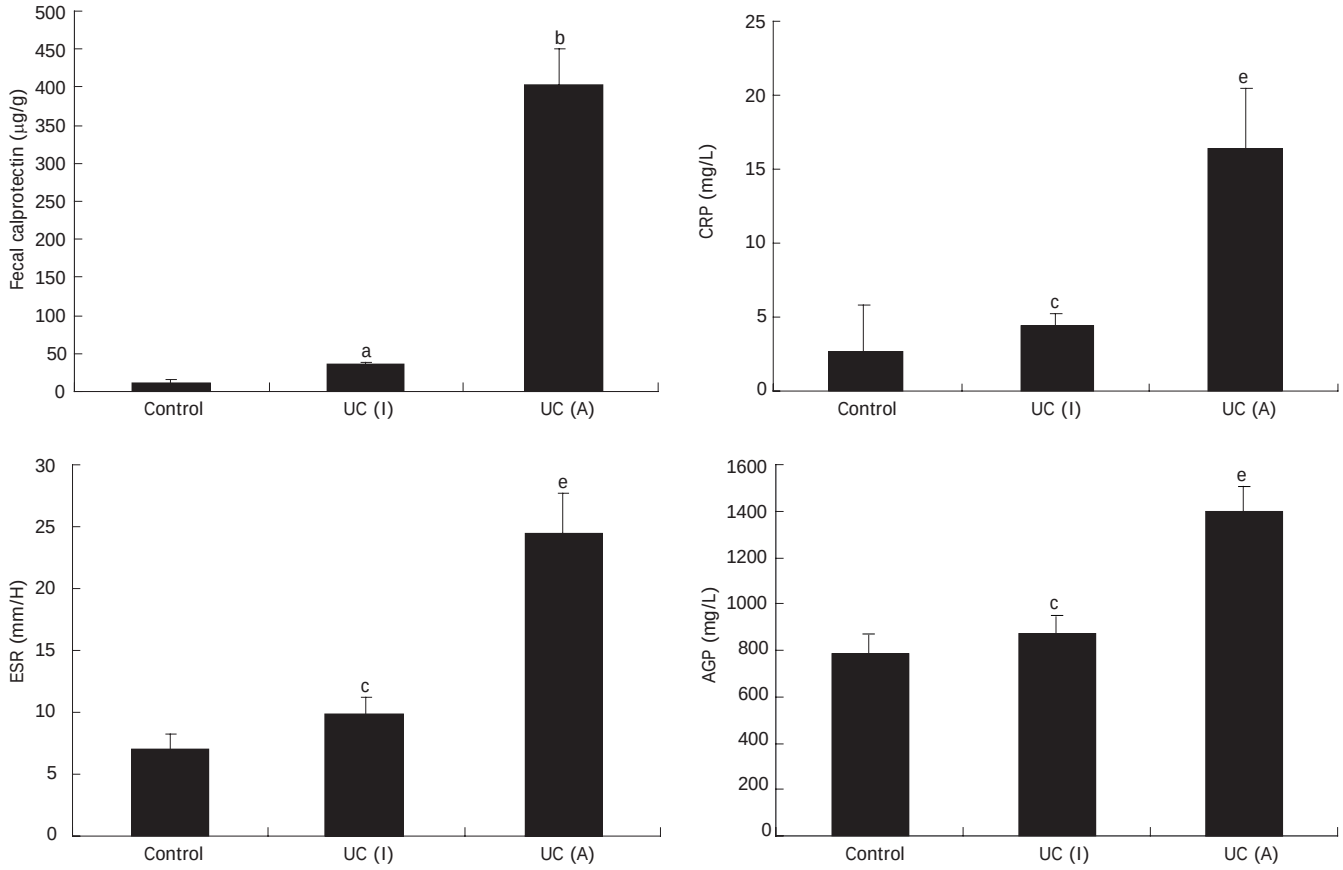


Figure 1 Concentrations of fecal calprotectin, CRP, ESR and AGP in the UC patients and the controls. UC (A): Ulcerative colitis (active phase); UC (I): Ulcerative colitis (inactive phase). [a]$P < 0.05$ *vs* the control, [b]$P < 0.01$ *vs* UC (I) and the control, [c]$P > 0.05$ *vs* the control, [e]$P < 0.05$ *vs* UC (I) and the control.

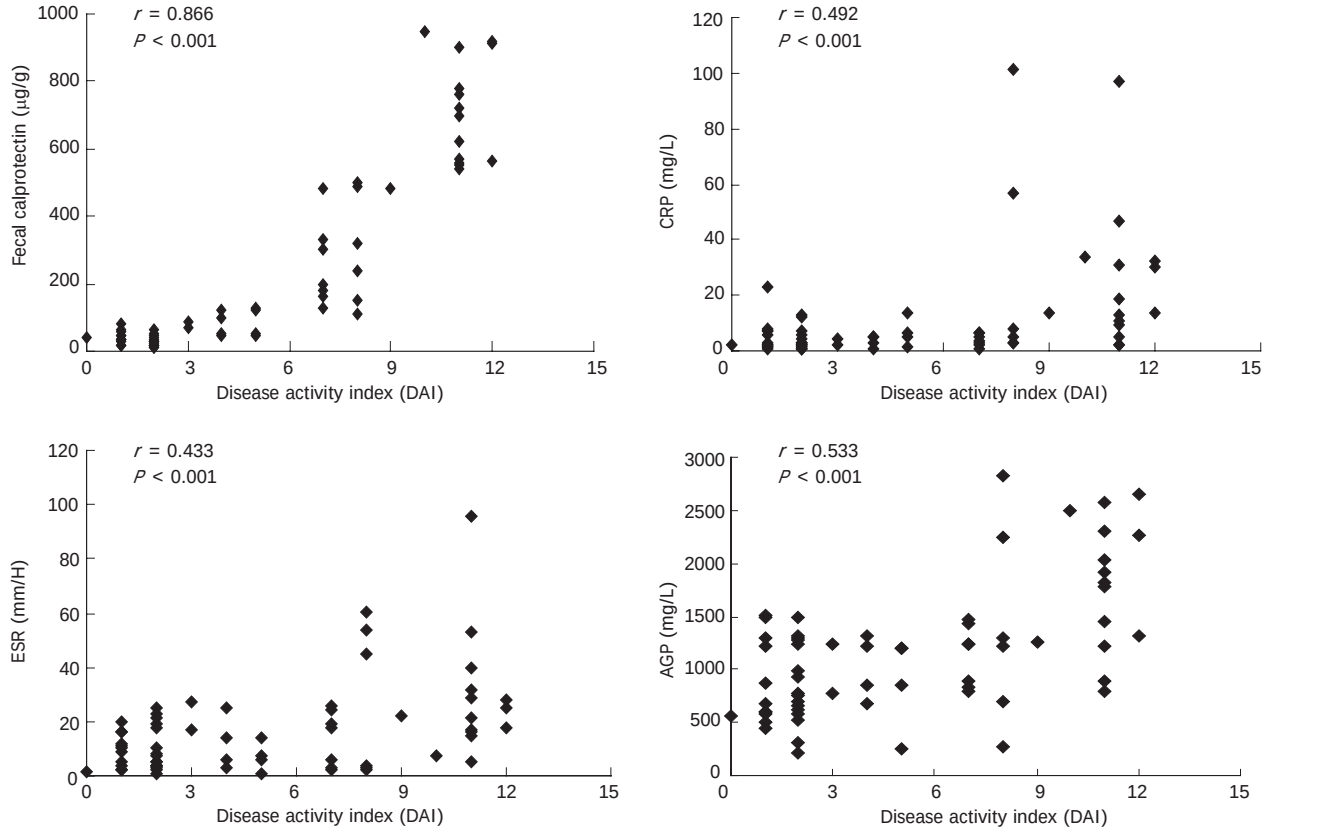


Figure 2 Concentrations of fecal calprotectin, CRP, ESR and AGP in UC and the DAI score of UC.

Table 1 Concentrations of fecal calprotectin, CRP, ESR and AGP in the UC patients and the controls (mean ± SD)

Group	Calprotectin (μg/g)	CRP (mg/L)	ESR (mm/H)	AGP (mg/L)
Control	11.5 ± 3.42	2.66 ± 3.2	7.05 ± 1.2	786.65 ± 77.65
Inactive UC	35.93 ± 3.39	4.39 ± 0.89	9.84 ± 1.36	870.14 ± 71.04
Active UC	402.16 ± 48.0	16.45 ± 3.98	21.44 ± 3.29	1394.9 ± 109.3

UC: Ulcerative colitis; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; AGP: Acid glycoprotein.

Table 2 Specificity and sensitivity for fecal calprotectin, CRP, ESR and AGP

Marker	Cut-off	Specificity (%)	Sensitivity (%)
Fecal calprotectin	50.0 μg/g	79.4	91.9
CRP	5.0 mg/L	69.0	62.2
ESR	15.0 mm/h	68.9	64.9
AGP	1200 mg/L	65.5	67.6

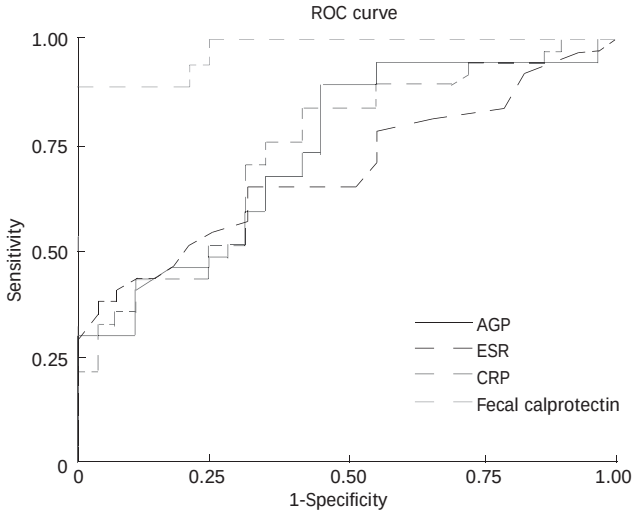


Figure 3 The ROC curve analysis on the abilities of calprotectin, CRP, ESR and AGP to make a difference between active UC and inactive UC.

between nought and three, making 12 the worst score): stool frequency, rectal bleeding, mucosa appearance, and physician's global assessment[27]. It reflects the clinical representation of UC and is the most extensive and simplest DAI. It is adopted by the guidelines for the management of inflammatory bowel disease in the fourth Asia Pacific Digestive Week[28]. Our results showed that fecal calprotectin concentrations were significantly higher in the patients with active UC than in the patients with inactive UC and in the controls. In addition, our results showed that fecal calprotectin concentration was higher in the patients with inactive UC than in the controls. In the patients with UC, the fecal calprotectin concentration had a better correlation with DAI than the CRP, ESR or AGP concentration.

The ROC curve is adopted not only to ensure the right cut-off point, but also to compare the diagnostic values of two or more diagnostic tests. The ROC analysis on the fecal calprotectin concentrations under these circumstances, showed that a cut-off point of 50.0 μg/g for calprotectin had a 91.9% sensitivity and a 79.4% specificity for making a differentiation between active UC and inactive UC. These were significantly better than those obtained with CRP, ESR and AGP.

In this study, ELISA was used to determine the fecal calprotectin concentrations in the patients with UC and in the controls. In comparison to endoscopy this method is simple, noninvasive and inexpensive. However, fecal calprotectin can only reflect the excretion of neutrophils. Many infective diseases can cause a large number of neutrophils to infiltrate, so that fecal calprotectin is elevated in a number of organic gastroenterological disorders[29-31]. Therefore, fecal calprotectin is not desirable as a method that is required to differentiate efficiently between UC and infective colitis, so it cannot take the place of an endoscope in diagnosing UC. Regardless of how sensitive the calprotectin technique may be in the detection of disease activity in patients with previously diagnosed UC, its greater potential use is in identifying patients with UC and in differentiating between patients with UC and patients with non-inflammatory disorders, still requires further study.

In conclusion, the determination of fecal calprotectin is an objective approach to grading the mucosal disease activity in patients with inflammatory bowel disease. The advantages of fecal calprotectin are simplicity, noninvasiveness, and relatively low cost. Fecal calprotectin can be useful not only in research but also in clinical practice.

COMMENTS

Background

The incidence and the prevalence rates of ulcerative colitis (UC) are increasing in China. It is important to accurately evaluate intestinal mucosa inflammation in the management of these patients, particularly for the assessment of therapeutic effectiveness. No clinical evaluations are specific for gut inflammation. Therefore, a new marker that will be more sensitive and specific for determination of disease activity of UC is urgently needed in clinical practice.

Research frontiers

Calprotectin can resist metabolic degradation caused by intestinal bacteria, and the protein is relatively stable in stools for up to one week at room temperature. Some foreign research has shown that fecal calprotectin concentrations were significantly higher in patients with active UC than in patients with inactive UC and in the controls.

Innovations and breakthroughs

Our study improved the originality of the manuscript assessing the relationship between calprotectin level and extension of colitis. This question was not described enough in the medical literature.

Applications

The aim of our study was to investigate the possibility and clinical application of fecal calprotectin in determining disease activity of UC. Our findings suggest that calprotectin in the patient's feces can reflect disease activity of UC and can be used as a rational fecal marker for intestinal inflammation in clinical practice.

Terminology

Receiver operating characteristics (ROC) curve is adopted not only to ensure the right cut-off point but also to compare the diagnostic values of two or more diagnostic tests. The accuracy of diagnostic test is characterized by the sensitivity and specificity. A ROC curve displays the sensitivity of a diagnostic test over all possible false-positive rates.

Peer review

This manuscript provided compelling evidence that fecal calprotectin was a better marker than C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) or acid glycoprotein (AGP) and improved the originality of the manuscript assessing the relationship between calprotectin level and extension of colitis. It deserves to be published.

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RAPID COMMUNICATION

Selection of treatment modality for hepatocellular carcinoma according to the modified Japan Integrated Staging score

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Abstract

AIM: To compare the prognosis of patients who underwent hepatectomy and ablation using the modified Japan Integrated Staging score (mJIS).

METHODS: We examined the clinicopathologic records and patient outcomes in 278 HCC patients including 226 undergoing hepatectomy and 52 undergoing ablation therapy.

RESULTS: Cirrhosis was more frequent in the ablation group. Tumor size, number and presence of vascular invasion were significantly higher in the operation group compared to the ablation group. The local recurrence rate adjacent to treated lesions was significantly higher in the ablation group compared to the operation group ($P < 0.05$). The 3- and 5-year survival rates in the ablation and the operation group were 66% and 78%, and 50% and 63%, respectively, but not significantly different. Over 50% survival rates were observed in patients with a mJIS score of 0-2 in both groups. However, survival rates with a score of 3-5 in both groups were significantly lower.

CONCLUSION: According to the mJIS system, both local treatments could be selected for patients with a score of 0-2. However, for patients with a score more than 3, liver transplantation might be a better option in patients with HCC.

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Key words: Hepatocellular carcinoma; Hepatectomy, Ablation; Modified Japan integrated staging score; Liver transplantation

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INTRODUCTION

Although hepatic resection is supposed to be the best curative local treatment for hepatocellular carcinoma (HCC)[1-3], sufficient hepatic functional reserve is necessary. In recent years, various treatment modalities have become available for HCC patients and the appropriate treatment should be selected according to tumor staging and liver function[4]. In the past decade, combined staging systems with tumor factors and liver function in HCC patients have been proposed worldwide[4-7]. We also proposed the modified cancer of the liver Italian program (mCLIP) score and the modified Japan Integrated Staging score (mJIS)[8,9]. Comparing the various staging systems using multivariate survival analysis, mJIS is the best available system to predict survival in HCC patients after hepatectomy[10]. The Liver Cancer Study Group of Japan subsequently showed that the mJIS system had good predictive accuracy for survival of Japanese patients with HCC by the records of 42269 patients diagnosed with HCC registered between 1992 and 1999 in a nationwide Japanese database[11].

Liver transplantation (LT) has recently been considered a good option to cure some HCC patients with poor hepatic function, such as Child-Pugh C cirrhosis[12]. The usefulness of cadavaric liver transplantation (LT) for HCC treatment has been clarified in Western countries[13] and, furthermore,

Todo *et al* reported good results in HCC treatment with the living related LT in Japan[14]. Therefore, LT seems to be a better treatment option in some patients who undergo local treatments in Japan. At this stage, Milan or University of California, San Francisco (UCSF) criteria using tumor factors and the Barcelona Clinic Liver Cancer (BCLC) system using tumor, liver function and performance status, have been used to decide the indication of LT[4,15,16]. It has not yet been clarified whether the mJIS system could be useful in the selection of LT in HCC patients.

In this study, we compare patient demographics, preoperative liver function, tumor parameters and long-term patient survival prognosis of 278 HCC patients who underwent hepatectomy and ablation using mJIS at several cancer institutions in Nagasaki prefecture, Japan. We then discuss the selection of treatment by comparing results by LT. Our aim is to clarify the treatment selection criteria for HCC patients using mJIS.

Table 1A Definition and criteria of the TNM stage for HCC according to the Liver Cancer Study Group of Japan[17]

Factor of T category	
1 Number of tumors: 1	
2 Tumor size: no more than 2 cm	
3 No vascular or bile duct invasion	
T category	T1: Fulfilling all three factors
	T2: Fulfilling two factors
	T3: Fulfilling one factor
	T4: Fulfilling none of the factors
N category	N0: Absence of lymph node metastasis
	N1: Presence of lymph node metastasis
M category	M0: Absence of distant metastasis
	M1: Presence of distant metastasis
Stage Ⅰ	T1 N0 M0
Stage Ⅱ	T2 N0 M0
Stage Ⅲ	T3 N0 M0
Stage Ⅳ-A	T4 N0 M0 or T1-T4, N1M0
Stage Ⅳ-B	T1-4, N0 or 1, M1

MATERIALS AND METHODS

Patients

We analyzed 278 patients with HCC who underwent surgical resection or ablation treatments in the Division of Surgical Oncology and the First and Second Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences (NUGSBS), and its related hospitals between 1994 and 2005. The study design was approved by the Human Ethics Review Board of our institution. Informed consent for data collection was obtained from each patient during this period. Anesthetic and patient data were retrieved from the NUGSBS database. Tumor stage and curability were determined according to the *Classification of Primary Liver Cancer*[17]. Subjects were divided into two groups: (1) Operation group with 226 patients. Preoperative treatment was performed in 78 patients including chemoembolization in 69 and thermal ablation in nine. Operative procedures included hemihepatectomy in 56 patients, anatomical sectriectomy in 74 and partial resection in 96. (2) Ablation group with 52 patients, including alcohol injection in 15 patients, radio-frequency ablation (RFA) in 32, and microwave coagulation therapy (MCT) in five patients.

Treatment indications, procedures and follow-up

The volume of liver to be resected was estimated according to results of the indocyanine green retention rate at 15 min (ICG R15) using Takasaki's formula[18]. Furthermore, hepatic function for hepatectomy was limited as ICGR15 < 40%, Child-Pugh classification A or B, and total bilirubin level < 2 mg/dL according to Miyagawa's criteria[19]. The expected liver volume for resection, excluding the tumor (cm^3), was measured by computed tomography (CT) volumetry[20]. Radical hepatectomy was performed to remove the hepatic tumor without leaving any residual tumor. The indications for hepatic resection of the size and the number of HCC were more than 2 cm, and less than or equal to three lesions, respectively. Distant metastasis was an extra-indication for hepatectomy. The assessment of tumor factors in the operation group was confirmed by histopathological examination of the resected specimen. We used the histopathological factors and curability by hepatectomy of the *Liver Cancer Study Group of Japan* by the *Classification of Primary Liver Cancer*[17].

For RFA or MCT, the indication for hepatic resection of the size and the number of HCC were less than 3 cm, and less than or equal to three lesions, respectively[21]. Hepatic function for ablations is limited as Child-Pugh classification A or B, platelet counts more than 50 000/mm^3, prothrombin activity more than 50% and total bilirubin level less than 3 mg/dL. When the appropriate coagulation was estimated to be incomplete by percutaneous puncture, ablations under laparoscopy, thoracoscopy or laparotomy were selected[22,23]. Evaluation of vascular involvement was performed by image analysis, such as enhanced computed tomography or magnetic resonance imaging.

After discharge from hospital, the patient status, laboratory data, and disease recurrence were checked every two to three months.

Staging criteria of the modified Japan Integrated Staging score (mJIS)

The assessment of each factor was confirmed by histopathological examination of the resected specimen, or by computed tomography scan, ultrasonography, magnetic resonance image or angiography. We used the pathological tumor-node-metastasis (pTNM) classification system of the Liver Cancer Study Group (LCSG) of Japan in 2000[17]. T category is determined by three factors of number, size and vascular or bile duct invasion. N category is the presence of lymph node metastasis and M category is the presence of distant metastasis. TNM staging has four stages according to T, N and M categories (Table 1A). Classification of Child-Pugh[24] and liver damage grade by LCSG[17] are shown in Table 1B. The original JIS score proposed by Kudo *et al* comprised the sum of points for two variables of the Japanese TNM classification and Child-Pugh classification[6]. In the modified JIS score proposed by our institute[9,10], the Child-Pugh classification score was replaced by that of liver damage grade by the LCSG of Japan (Table 1C).

Table 1B Definition and criteria of Child-Pugh classification and liver damage grade

Child-Pugh classification[24]	A	B	C
Encephalopathy	none	mild	coma
Ascites	none	responsive	unresponsive
Serum bilirubin (mg/dL)	< 2.0	2.0-3.0	> 3.0
Serum albumin (g/dL)	> 3.5	2.8-3.5	< 2.8
Prothrombin activity (%)	> 70	40-70	< 40
Liver damage grade[17]	A	B	C
Ascites	none	responsive	unresponsive
Serum bilirubin (mg/dL)	< 2.0	2.0-3.0	> 3.0
Serum albumin (g/dL)	> 3.5	3.0-3.5	< 3.0
ICG R15 (%)	< 15	15-40	> 40
Prothrombin activity (%)	> 80	50-80	< 50

Table 1C Definition and criteria of the JIS and the mJIS

	Score			
	0	1	2	3
Original JIS score[6]				
Japanese TNM stage	I	II	III	IV
Child-Pugh Classification	A	B	C	
Modified JIS score[9]				
Japanese TNM stage	I	II	III	IV
Liver damage grade	A	B	C	

TNM: Tumor-node metastasis.

Statistical analysis

Continuous data were expressed as the mean ± SD. Data from different groups were compared using one-way analysis of variance (ANOVA) and examined by student's *t*-test or Dunnet's multiple comparison test. For univariate analysis, categorical data were analyzed by the Fisher's exact test. Disease-free and overall survival rates were calculated according to the Kaplan-Meier method, and differences between groups were tested for significance using the log-rank test. Multivariate analysis was performed using the proportional hazards regression model. A two-tailed *P* value of < 0.05 was considered significant. Statistical analyses were performed using SAS software (Statistical Analysis System Inc., Cary, NC).

RESULTS

Patient age, gender and period of treatment were not significantly different between groups (Table 2). Rates of cirrhosis and Child-Pugh B were significantly higher in the ablation group. Thirty-five percent of patients underwent pretreatment in the operation group; however, no patients underwent other treatments in the ablation group. Tumor size in the operation group was significantly larger than that in the ablation group. The number of tumors and rate of vascular involvement were significantly higher in the operation group compared to the ablation group. Posttreatment adjuvant treatments were similarly performed in both groups.

In the ablation group, tumor relapse was observed in 22 patients (42%) including 10 with intrahepatic metastasis and 12 with local recurrence adjacent to the ablated site (Figure 1). On the other hand, in the operation group, tumor relapse was observed in 122 patients (62%), which included 117 with intrahepatic metastasis and five with local recurrence adjacent to the resected margin. The local recurrence rate adjacent to the treated lesion was significantly higher in the ablation group compared to the operation group ($P < 0.05$).

By applying mJIS, discrimination of survival in each score was remarkable (Figure 2). The 3- and 5-year survival rates in the ablation and operation group were 66% and 78%, and 50% and 63%, respectively (Figure 3); however, there were no significant differences between groups. Child-Pugh B was significantly associated with poor disease-free and overall survival (Table 3). Multiple tumors were associated with overall survival. However, difference of treatment modality was not associated with prognosis in the present study. Table 4 shows 3-year survival rates in each score of mJIS; however, there were no significant

Table 2 Patient demographics between two groups in HCC patients

	Operation (*n* = 226)	Ablation (*n* = 52)	*P*-value
Age (yr)	60.2 ± 10.5	58.3 ± 10.7	0.074
Gender			
male/female	179/43	37/15	0.283
Time to treatment (yr)[1]	(5.1, 8.4, 11.2)	(5.4, 9.3, 11.6)	0.28
Background liver			
chronic hepatitis/cirrhosis/normal	119/94/13	4/48/0	< 0.001
Hepatitis virus			
B/C/B&C/non-B non-C	72/116/11/27	11/36/5/0	0.007
Child-Pugh classification			
A/B	201/25	34/18	< 0.001
Pretreatment			
Yes/No	78/148	0/52	< 0.001
Tumor size			
< 5 cm/ ⩾ 5 cm	160/66	49/3	< 0.001
Number of tumors			
solitary/multiple	174/52	37/15	0.479
Vascular involvement			
No/Yes	162/64	48/4	0.003
Adjuvant therapy			
Yes/No	5/221	0/52	0.615

[1]Each triplet gives the 25th, 50th and 75th sample percentiles. Time to the treatment since 1 January 1994.

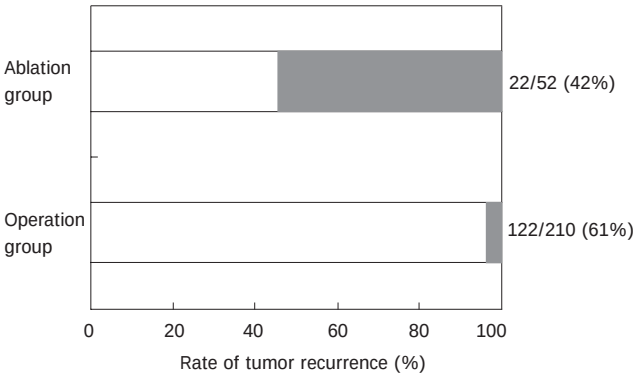


Figure 1 Tumor relapse and site of recurrence after treatment in two groups. Open square shows intrahepatic metastasis and closed square shows the local recurrence adjacent to treated lesion.

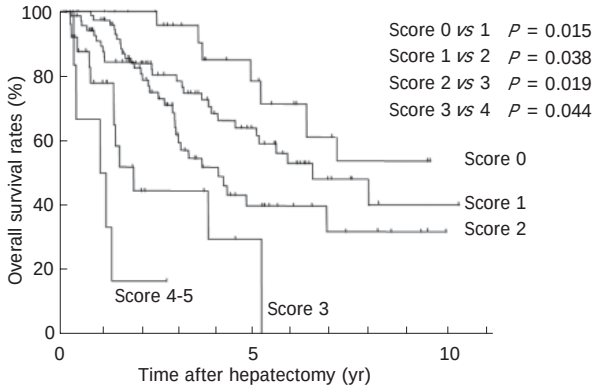


Figure 2 Survival using mJIS in HCC patients who underwent hepatic resection.

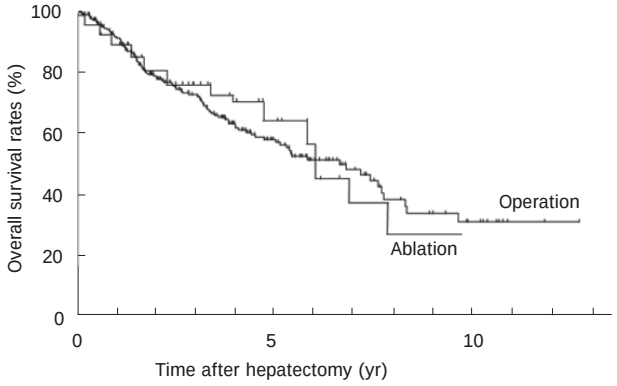


Figure 3 Comparison of survival in HCC patients between operation and ablation groups.

Table 3 Survival between two groups in HCC patients by multivariate analysis

	Disease-free survival		Overall survival	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Child-Pugh classification				
B *vs* A	2.05 (1.25-3.35)	0.004	2.46 (1.38-4.41)	0.002
Tumor size				
⩾ 5 *vs* < 5 cm	1.26 (0.83-1.92)	0.282	1.30 (0.75-2.25)	0.350
Macroscopic findings[1]				
Confluent type *vs* Simple nodular type	1.10 (0.75-1.61)	0.626	1.58 (0.92-2.72)	0.098
Number of tumor				
Multiple *vs* Solitary	1.23 (0.82-1.85)	0.306	1.73 (1.03-2.91)	0.037
Vascular involvement				
Yes *vs* No	1.24 (0.68-2.27)	0.481	1.76 (0.93-3.31)	0.080
Alpha-feto protein level				
⩾ 400 *vs* < 400 ng/mL	1.41 (0.93-2.11)	0.103	1.36 (0.81-2.28)	0.250
Treatment modality				
Ablation *vs* Hepatectomy	0.75 (0.46-1.22)	0.239	0.64 (0.39-1.34)	0.334

[1]Macroscopic findings defined by the *Classification of Primary Liver Cancer*[17].

Table 4 The 3-year survival rates in each score of mJIS after treatments in HCC patients

	Operation group (*n* = 226) (%)	Ablation group (*n* = 52) (%)
mJIS 0	96	98
mJIS 1	80	73
mJIS 2	66	70
mJIS 3	39	48
mJIS 4	35	28
mJIS 5	25	-

differences between the two groups. Overall survival rates between score 2 and 3 were remarkably different in both groups. Over 50% survival rates were obtained up to score 2 in both groups; however, survival rates over score 3 in both groups were lower.

DISCUSSION

With recent advances in the ablation technique, local tumor control has improved[25]. In comparison with alcohol injection, the modality option or patient survivals have been remarkably improved in the era of RFA or MCT[25-28]. Alcohol injection is not recommended at present[29,30]. In the present study, the ablation group included patients undergoing alcohol injection; however, local control was relatively good because of the small HCC in our series. At this stage, we mainly performed RFA regardless of tumor size, number and location and we also applied RFA under laparotomy or laparoscopy to achieve complete ablation. Selection bias for treatment was shown by our results. Hepatectomy was mainly selected in patients with chronic hepatitis or Child-Pugh A, while ablation was used in patients with impaired liver function such as cirrhosis or Child-Pugh B. In the latter, surgical resection is usually avoided. Concerning tumor factors, hepatectomy was preferably selected for tumors of larger size, and solitary and vascular involvement by the image examinations. Ablation tended to be selected for small and multiple tumors. Wakai *et al* also showed a similar tendency to select the treatment modality[31]. However, Shiina *et al* described the superiority of RFA compared to hepatectomy[32]. Therefore, superiority or selection criteria between both treatments are still controversial. In the recent Japanese guidelines for HCC, the indication of these treatments was not clearly discriminated[33]. In the operation group, other treatments were preferably performed in one-third of all patients. In these patients, ablation therapy was included as well. Based on these results, the background in both groups was remarkably different, which was also the case in Wakai's report[31].

The pattern of tumor relapse was different in the present study and local recurrence adjacent to the ablated section was significantly higher regardless of careful ablations with a sufficient ablation margin more than 5 mm[34]. Some investigators reported that the complete ablation rate is around 90% with HCC and less than 5 cm could be treated[27-29,35]. However, local recurrence in patients undergoing thermal ablation therapy ranged between 9.2% and 13.6%[26-28]. Hong *et al* reported that the local recurrence rate in ablation therapy was higher than that in hepatic resection[36]. Therefore, local control by hepatectomy is superior to that by thermal ablation at this stage, based on the above reports[26-28,31,35] and our results. Although the rate of distant liver metastasis might not be remarkably different based on previous reports[26-28,35,36], tumor recurrence in the distant liver was still high in the operation group in the present study, which might be

associated with the advanced stage of HCC as shown in the results.

With respect to patient survival after treatment, superiority between both groups was not clarified, in addition to survival rate, in our results. We applied the mJIS system in this study, which is the best available to predict HCC patient survival after curable treatments[9-11]. By applying this system, survival in each score in the present series was well discriminated. At this stage, indication of treatment modality in HCC patients with early tumor stage and Child-Pugh A or B between hepatectomy and thermal ablation has been controversial[31,32,34,37]. In patients with small HCC less than 2 cm or in patients with impaired liver function such as a Child-Pugh B, survival benefit was similar between both groups[31,34,37,38]. As described above, local recurrence rate by thermal ablation was higher compared to that by hepatectomy; however, overall survival was not significantly different by previous reports[26-28,34-37]. The guideline for diagnosis and treatment in HCC patients was first proposed by Makuuchi *et al*[33]; however, ablation and hepatectomy were at similar locations in HCC patients with less than four sites and good liver function. Our results showed that the overall survival was similar between both groups, which also had similar mJIS scores, although the included tumor factor and liver function were different between groups, as shown in patient demographics. By multivariate analysis, the difference of treatment modality was not observed in the present study. Up to mJIS score 2, the 3-year survival rate was well maintained; however, survival rate over mJIS score 3 was significantly decreased in both groups. We considered that this border between a score of 2 and 3 might be important to decide the limitation of both conventional treatments. The ultimate curable option should be a liver transplantation. Todo *et al* reported posttransplant survival in HCC patients who met Milan criteria undergoing living-related liver transplantation in Japan[14]. The 3-year survival rates with or without Milan criteria was 79% and 60%, respectively. This report was a satisfactory result at this stage. Compared to this result, even in a group with Milan criteria not met, survival rates over score 3 in both groups were lower than those in HCC patients undergoing living-related LT. In HCC patients with mJIS score 3-5, patients without remarkable vascular involvement would be included in the indication of liver transplantation. By comparing the survival benefit, transplantation is strongly recommended to improve patient prognosis. In patients with score 0-2, the definition of treatment criteria between groups seemed to be difficult on the present evidence and consensus[39,40]. A study with a large series in Japan will clarify this problem in the near future. Our results did not show superiority or definite indication between both treatment modalities in the present study. To improve the survival results in HCC patients, a combination of both treatments[41], chemoembolization[42], or intra-operative ablation under laparoscopy or laparotomy should be used[22,43].

In conclusion, hepatectomy tended to be selected in patients with better functional liver reserve and, hence, ablation therapy tended to be selected in patients with poor hepatic function in our series. In the ablation group, local recurrence near the treatment region tended to be more than that in the hepatectomy group. By multivariate analysis, macroscopic finding and vascular invasion were significant risk factors, but treatment modality was not a prognostic factor. According to the mJIS system, both treatments could be selected for patients with score 0-2; however, for patients with a score more than 3, liver transplantation might be a better option compared to conventional local treatments.

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RAPID COMMUNICATION

Role of 18F-fluorodeoxyglucose positron emission tomography imaging in surgery for pancreatic cancer

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Abstract

AIM: To evaluate the role of positron emission tomography using 18F-fluorodeoxyglucose (FDG-PET) in the surgical management of patients with pancreatic cancer, including the diagnosis, staging, and selection of patients for the subsequent surgical treatment.

METHODS: This study involved 53 patients with proven primary pancreatic cancer. The sensitivity of diagnosing the primary cancer was examined for FDG-PET, CT, cytological examination of the bile or pancreatic juice, and the serum levels of carcinoembrionic antigens (CEA) and carbohydrate antigen 19-9 (CA19-9). Next, the accuracy of staging was compared between FDG-PET and CT. Finally, FDG-PET was analyzed semiquantitatively using the standard uptake value (SUV). The impact of the SUV on patient management was evaluated by examining the correlations between the SUV and the histological findings of cancer.

RESULTS: The sensitivity of FDG-PET, CT, cytological examination of the bile or pancreatic juice, and the serum levels of CEA and CA19-9 were 92.5%, 88.7%, 46.4%, 37.7% and 69.8%, respectively. In staging, FDG-PET was superior to CT only in diagnosing distant disease (bone metastasis). For local staging, the sensitivity of CT was better than that of FDG-PET. The SUV did not correlate with the pTNM stage, grades, invasions to the vessels and nerve, or with the size of the tumor. However, there was a statistically significant difference (4.6 ± 2.9 *vs* 7.8 ± 4.5, $P = 0.024$) in the SUV between patients with respectable and unresectable disease.

CONCLUSION: FDG-PET is thus considered to be useful in the diagnosis of pancreatic cancer. However, regarding the staging of the disease, FDG-PET is not considered to be a sufficiently accurate diagnostic modality. Although the SUV does not correlate with the patho-histological prognostic factors, it may be useful in selecting patients who should undergo subsequent surgical treatment.

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Key words: Pancreatic cancer; Fluorodeoxyglucose positron emission tomography; Computed tomography; Standard uptake value; Carcinoembrionic antigens; Carbohydrate antigen 19-9; Prognostic factor

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INTRODUCTION

Today, despite advances in diagnosing modalities, most patients with pancreatic cancer are still unresectable at the time of diagnosis. Surgery remains the only potential for long-term survival, with a resectability rate of around 15%-20% in the latest review[1]. Even in patients with resectable disease, the 5-year survival rate is still around 20%[1-3]. For patients with unresectable disease, chemotherapy with or without radiation therapy is usually chosen, and the survival benefit of chemotherapy with Gemcitabine over 5-fluorouracil has recently been reported[4]. Clearly, an effort has to be made for diagnosing early stage cancer, also by determining its clinical stage and by accurately predicting the prognosis, thus avoiding unnecessary surgical explorations.

Recently, the advantages of positron emission tomography using 18F-fluorodeoxyglucose (FDG-PET) on diagnosing pancreatic cancer, especially small lesions less than 2 cm in size, over the conventional modalities, including computed tomography (CT), have been reported[2]. Furthermore, FDG-PET has also been reported to possibly play a role in predicting the prognosis of pancreatic cancer[2,5,6]. However, the significance of FDG-PET imaging in the management of pancreatic cancer, including diagnosis, staging, or

predicting prognosis, has not yet been established. In this study, we explored the role of FDG-PET in surgery for pancreatic cancer by examining its sensitivity in diagnosing and staging, and its potential for predicting prognosis by comparing the standard uptake value (SUV) and the histological findings.

MATERIALS AND METHODS

Patients

This study involved 53 patients with histologically (30 patients) or clinically (23 patients) proven primary pancreatic cancer who had undergone FDG-PET from January 2004 to January 2007. In clinically proven pancreatic cancer patients metastatic lesions were detected in the liver, lymph nodes, or the other organs, by the following up CT retrospectively, and the patients died of the primary pancreatic cancer. The patients' mean age was 70.1 years (range from 44 to 84 years). The patients consisted of 33 males and 20 females. The localisation of the cancer was in the head of the pancreas in 32 patients and in the body and tail in 21 patients. Twenty-eight patients presented with diabetes requiring insulin therapy. Among the 53 patients, 28 patients were diagnosed to be unresectable, and 25 patients eventually underwent surgery with a curative intention, although in 7 of them the cancer turned out to be unresectable because of the intraoperative findings.

Methods

The sensitivity of diagnosing pancreatic cancer was examined for FDG-PET, CT, cytological examination of the bile or pancreatic juice, and the serum levels of carcinoembrionic antigens (CEA) and carbohydrate antigen 19-9 (CA19-9). Next, the accuracy of staging of the disease and the impact on patient management was evaluated and compared between FDG-PET and CT. Finally, FDG-PET was analyzed semiquantitatively using the standard uptake value (SUV). In the patients who underwent surgery, a correlation was found between the preoperatively obtained SUV and the histological findings of the specimen in regard to the pathological stagings of cancer classified based on the UICC TNM classification (6th edition, 2002), histological differentiation, lymphatic invasions, vascular invasion, and intrapancreatic nerve invasion. The SUV was compared between the resectable and unresectable disease patient groups. A correlation between the SUV and the maximum diameter of the primary lesion, which was determined on CT, was examined. All of the studies were performed retrospectively by collecting and analyzing data from the patient records.

Cytological examination of the bile or pancreatic juice

The bile or pancreatic juice, which was collected from 28 patients after brushing endoscopic retrograde cholangiopancreatography (ERCP), was subjected to a cytological examination. This was performed using the Papanicolaou staining, and the diagnosis established according to the Papanicolaou classification. The results in Classes Ⅰ, Ⅱ, or Ⅲ were counted as negative in this study; the results in Classes Ⅳ or Ⅴ were counted as positive.

CT

For multidetector CT scans, a contrast enhancement was performed for each patient. Helical images of the abdomen were routinely obtained and reconstructed with 5 mm thickness. The CT images were interpreted independently and consecutively by two radiologists with extensive experience of more than 10 years in CT scanning. The findings of the CT scans were considered positive when both of the radiologists strongly suspected malignant disease due to a discrete low-attenuation mass within the pancreas, and/or involving the adjacent vessels, and/or swelling of the regional lymph nodes.

FDG-PET

The FDG-PET images were acquired with PET machine (Siemens EXACT HR+, CTI, Knoxville, TN, USA). The patients were required to fast for at least 4 h before PET imaging. One hour after the intravenous administration of 5 mCi of FDG, the emission images were acquired. The transmission images were acquired to correct for attenuation. The FDG-PET images were interpreted independently and consecutively by two radiologists with extensive experience in FDG-PET imaging. The findings were considered to be positive when both of the radiologists strongly suspected malignant disease. In addition, the images were analyzed semiquantitatively using the SUV, as reported elsewhere[7]. Briefly, regions of interest measuring 1.0 cm^2 were drawn over the area of maximum activity in a lesion. The SUV was calculated as follows:

SUV = (activity in region of interest in mCi)/(injected dose in mCi/weight in kg).

Statistical analysis

The chi-square test was employed for a statistical comparison of the sensitivity of FDG-PET and CT. The Student's *t* test was used to compare the values of the SUV between the two groups. Finally, correlations between the SUV and the maximum diameter of the primary lesion determined on CT were examined by the Pearson's correlation test. All statistical analyses were performed using the SPSS software program (SPSS, Chicago, USA). A P value < 0.05 was considered statistically significant.

RESULTS

Sensitivity for diagnosing pancreatic cancer

The sensitivity of FDG-PET, CT, cytological examination of the bile or pancreatic juice, and the serum levels of CEA and CA19-9 were 92.5% (49/53), 88.7% (47/53), 46.4% (13/28), 37.7% (20/53) and 69.8% (37/53), respectively.

Sensitivity for preoperative staging

Among the 53 patients, 28 patients were diagnosed to be unresectable based on the following preoperative imaging findings: invasion to major arteries such as the supramesenteric artery, celiac trunks, or common hepatic artery in 9 patients, para-aortic lymph node metastasis in 12 patients, detection of hepatic metastasis in 14 patients,

Table 1 Histological findings of the patients

Histological findings			Number of patients	SUV (mean ± SD)	Difference
Pathological stage (TNM)					
pT	pN	stage			
1	0	ⅠA	1	3.59	
1	1	ⅠB	1	3.14	
3	0	ⅡA	8	4.1 ± 0.9	NS
1, 2, 3	1	ⅡB	8	5.9 ± 4.5	
Histological differentiation					
Well			7	3.7 ± 0.7	NS
Moderately-poorly			11	5.6 ± 3.6	
Lymphatic invasions					
Negative			9	5.3 ± 3.9	NS
Positive			9	4.5 ± 2.1	
Venous invasions					
Negative			6	6.2 ± 5.1	NS
Positive			12	4.4 ± 1.9	
Intrapancreatic nerve invasions					
Negative			11	4.8 ± 3.5	NS
Positive			7	5.1 ± 2.3	

SUV: Standard uptake value; NS: Not significant.

detection of bone metastasis in 7 patients, strongly suspected peritoneal dissemination in 5 patients, general complications associated with aging in 4 patients. Among the 25 patients who eventually underwent surgery with a curative intention, the cancer turned out to be unresectable in 7 of them because of the following intraoperative findings: detection of hepatic metastasis (5 to 8 mm in diameter) in 5 patients, histologically confirmed metastases in the para-aortic lymph node (less than 1 cm in diameter) in 2 patients, and cytologically positive ascites in 2 patients. Therefore, the staging diagnostic sensitivity of FDG-PET and CT was 2/9 (22.2%) and 9/9 (100%) for invasion into the major arteries, 8/14 (57.1%) and 11/14 (78.6%) for para-aortic regional lymph nodes metastases, 10/19 (52.6%) and 14/19 (73.7%) for hepatic metastases, 8/8 (100%) and 1/8 (12.5%) for bone metastases, 3/7 (42.9%) and 4/7 (57.1%) for peritoneal dissemination, respectively. There was a statistically significant difference in the sensitivity detecting invasion into the major arteries ($P = 0.001$) and bone metastases ($P = 0.001$), based on the chi-square test.

Correlation between the SUV and histological findings

In 18 patients, a surgical resection with a lymphadenectomy was performed. A pancreaticoduodenectomy was performed in 11 patients, while a distal pancreatectomy was performed in 7 patients. In these patients, a pathohistological examination was performed. As Table 1 shows, no statistically significant difference was found in any of the histological findings listed in Table 1.

Comparison of SUV between resectable and unresectable disease

As Figure 1 shows, the SUV of the primary lesion was 4.6 ± 2.9 (mean ± SD) in the resectable disease cases, while it was 7.8 ± 4.5 in the unresectable cases, with a statistically significant difference ($P = 0.024$) by the Student's *t*-test. On the other hand, the maximum diameter of the primary lesion measured on CT was 27.9 ± 10.9 mm in the resectable cases, and 40.5 ± 21.4 mm in the unresectable ones ($P = 0.053$). Furthermore, we found no correlation between the SUV and the maximum diameter of the primary lesion measured on CT by using Pearson's correlation test ($P = 0.274$), as shown in Figure 2.

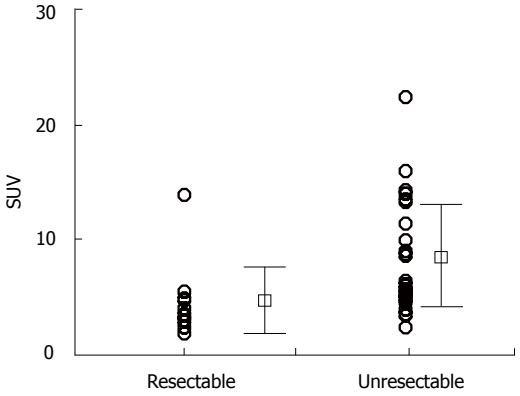


Figure 1 Comparison of the SUV of the primary lesion between patients with resectable and unresectable disease. SUV: Standard uptake value.

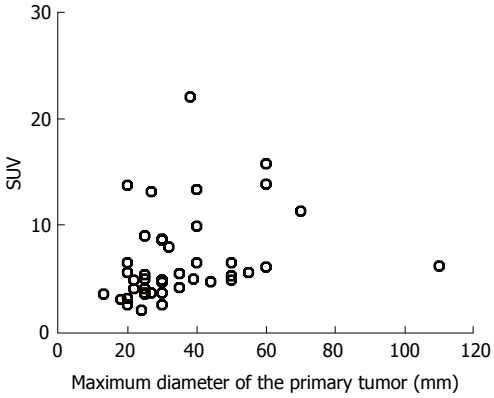


Figure 2 Relationship between the SUV and the maximum diameter of the primary lesion.

DISCUSSION

Pancreatic cancer is one of the most lethal human cancers and it continues to be a major unsolved health problem worldwide. Despite efforts in the past 50 years, conventional treatment approaches, such as surgery, radiation, chemotherapy, or combination of these, have had little impact on the course of this aggressive neoplasm[1]. Although recent progress in systemic chemotherapy has been reported[8], surgery remains the only hope for long-term survival[9]. In surgery for pancreatic cancer, a great deal of effort has been made to expand the resection with an extended lymphadenectomy in order to improve the outcome[10]. However, only patients with localized disease and a tumor size less than 2 cm with no lymph node metastases can expect long-term survival after surgery[1,2,11]. Therefore, increased efforts should be focusing not only on diagnosing the early stage disease, but also on staging and predicting prognosis so that unnecessary surgical exploration may be avoided.

Recently, PET has been reported to have superiority to CT, US, and EUS in its sensitivity and specificity in diagnosing pancreatic cancer[2,12]. Furthermore, regarding the role of PET in diagnosing the disease, the metabolic activity of the tumor may be of prognostic significance[5,6]. However, the role of PET in the management of pancreatic cancer has yet to be established.

The role of FDG-PET in diagnosis of primary cancer

The sensitivity of FDG-PET was 92.5%, and it was better than that of CT, cytological examinations of the bile or pancreatic juice, and the serum levels of CEA and CA19-9. Our findings also correlate with those of previous reports, in which the sensitivity of FDG-PET, CT, and US has been reported to be 94%, 89%, and 89%, respectively[12]. On the other hand, in another study, the sensitivity of FDG-PET was found to be lower than or equal to CT[13-15] and these findings thus remain controversial. In particular, when a multidetector CT is performed routinely with thin sections (1 mm), the sensitivity of the CT may further improve[2]. This study was conducted among patients with the histologically or clinically proven pancreatic cancer, and therefore the specificity was not evaluated.

The role of FDG-PET in staging of the disease

This study showed that, as far as staging is considered, FDG-PET was superior to CT only in diagnosing distant disease. On the other hand for local staging the sensitivity of CT was better than that of FDG-PET. As described[2], the poor spatial resolution of FDG-PET limits the local staging of pancreatic cancer. Therefore, the anatomical imaging modalities with CT are better suited to demonstrate the relationship of the tumor, adjacent organs, and vascular structure.

In the nodal staging of the disease, there was no difference between FDG-PET and CT, since both performed poorly. The reported sensitivities of FDG-PET have varied between 46% and 71%[2,16]; thus the results of this study are considered to correlate with these previous reports. It has been said that one possible reason for the apparent low sensitivity of FDG-PET is the close proximity of the peripancreatic and paraaortic lymph node basins to the primary tumor, which can obscure their detection[2,16].

The important impact of FDG-PET on staging has been in its ability to identify distant metastases[17]. According to the previous reports[18], the sensitivity of FDG-PET for detecting hepatic metastases is about 70%, while that of this study was 52.6%. In particular, small lesions less than 1 cm could not be detected. It has also been reported that the sensitivity for lesions less than or greater than 1 cm is 43% and 97%, respectively[19]. Direct spread into the peritoneum is also not uncommon and often is missed on conventional imaging. However, in this study, both FDG-PET and CT failed to demonstrate a high degree of accuracy in these diagnostic analyses.

The prognostic significance of FDG-PET

Some researchers have shown the SUV in FDG-PET to be an independent prognostic factor in a subpopulation of patients with pancreatic cancer. Nakata *et al*[5] showed that in patients with unresectable disease, a high SUV correlated with a shorter survival. Maemura *et al*[20] reported that pancreatic tumors with distant metastasis showed significantly higher SUV levels than tumors without metastasis. Sperti *et al*[6] also demonstrated that a high SUV (> 4.0) was associated with shorter survival. For prognostic factors for pancreatic cancer, the tumor stage and grade[21], R0 resection[21], levels of serum tumor marker[22], size of the primary lesion[3], and status of nodes metastases[3,11], have been reported. On the other hand, the increased glycolytic activity of the tumor detected by the SUV may represent tumor growth and also resemble the tumor's biological behavior[23-25]. Therefore, in this study we examined the correlation between the SUV and the patho-histological findings. The results were that the SUV did not correlate with the pTNM stage, grades, invasions to the vessels and nerve, or size of the tumor. Sperti *et al*[6] also found no difference between a high SUV (> 4.0) and a low SUV patients group in regard with TNM staging and histological grading, as shown in this study. Nevertheless, they found SUV to be an independent prognostic factor in patients with pancreatic cancer, and therefore advocated that the different biological aggressiveness of the tumor, detected by the SUV, might explain the difference in survival in patients with otherwise similar prognostic valuables. In the present study, there was a statistically significant difference in the SUV between the patients with resectable and unresectable disease. This may support the findings of previous reports which describe the SUV to be an independent prognostic factor. Therefore, although in staging FDG-PET did not perform precisely enough except for detecting bone metastases, by evaluating the SUV, FDG-PET may provide such additional information on the biological aggressiveness of the tumor, and thus play an important role in helping to select patients for subsequent surgical therapy. Some small studies reported that the tumor SUVs were useful in predicting the effectiveness of chemotherapy for unresectable pancreatic cancer[17,26,27]. However, conclusions must await further studies including larger population of patients.

In conclusion, FDG-PET was found to be useful for diagnosing pancreatic cancer. However, in staging of the disease, FDG-PET does not perform precisely enough. Although the SUV does not correlate with the patho-histological prognostic factors, it may be useful in selecting patients who should undergo the subsequent surgical treatment. Therefore, FDG-PET may play an important role in the decision making process and surgical management for patients with pancreatic cancer. However, this study suffers from the limitation of a small population of patients. Therefore, a definite conclusion may have to wait for further studies involving a larger population of patients. Furthermore, the use of image fusion with PET/CT[28,29] or with PET/MRI[30] may improve the accuracy when staging pancreatic cancer.

COMMENTS

Background

Pancreatic cancer is one of the most lethal human cancers and it continues to be a major unsolved health problem worldwide. Only patients with localized disease and

a tumor size less than 2 cm with no lymph node metastases can expect long-term survival after surgery. Therefore, increased efforts should be focusing not only on diagnosing the early stage disease, but also on staging and predicting prognosis so that unnecessary surgical exploration may be avoided. Recently, positron emission tomography (PET) has been reported to be superior to computed tomography (CT), ultrasound (US), and endoscopic US (EUS) in its sensitivity and specificity in diagnosing pancreatic cancer. Furthermore, the metabolic activity of the tumor evaluated by uptake value of fluorodeoxyglucose (FDG) (standardized uptake value: SUV) may be of prognostic significance. However, the role of PET in the management of pancreatic cancer has yet to be established.

Research frontiers

As is described above, pancreatic cancer is one of the most lethal human cancers and it continues to be a major unsolved health problem worldwide. Despite efforts in the past 50 years, conventional treatment approaches, such as surgery, radiation, chemotherapy, or combination of these, have had little impact on the course of this aggressive neoplasm. Although recent progress in systemic chemotherapy has been reported, surgery remains the only hope for long-term survival. In surgery for pancreatic cancer, a great deal of effort has been made to expand the resection with an extended lymphadenectomy in order to improve the outcome. However, only patients with localized disease and a tumor size less than 2 cm with no lymph node metastases can expect long-term survival after surgery. Therefore, breakthrough for pancreatic cancer may be achieved in diagnosing an early stage disease.

Innovations and breakthroughs

The present study showed that FDG-PET was useful for diagnosing pancreatic cancer. However, in staging of the disease, FDG-PET does not perform precisely enough. Although the SUV does not correlate with the patho-histological prognostic factors, it may be useful in selecting patients who should undergo the subsequent surgical treatment. Therefore, FDG-PET may play an important role in the decision making process and surgical management for patients with pancreatic cancer.

Applications

By using FDG-PET in combination with conventional diagnostic modalities, such as computed tomography (CT), ultrasound, magnetic resonance imaging, and endoscopic pancreatography, the ability to diagnose the early stage pancreatic cancer, or to select candidates for surgery can be improved. At the same time, as the increased glycolytic activity of the tumor detected by SUV may represent tumor growth and resemble the tumor biological behavior, the SUV may provide additional information on the biological aggressiveness of the tumor.

Terminology

FDG-PET: 18F-fluorodeoxyglucose (FDG) is a positron-emitting radio-tracer that is transported intracellularly *via* glucose transporters which are highly expressed in various cancer cells, then FDG is phosphorylated by hexokinase to FDG-6-PO4. However, further metabolism of FDG-6-PO4 is not possible in the neoplastic cells due to insufficient phosphatase levels and tracer accumulation. Therefore, FDG-PET can image cancer cells based on such specific tissue metabolism. SUV: To perform a quantitative analysis for accumulation of FDG, the standardized uptake value (SUV) is calculated in the suspected neoplastic foci. The SUV was calculated as follows:

SUV = (activity in region of interest in mCi)/(injected dose in mCi/weight in kg).

Peer review

This is a good clinical research on the diagnosis, staging and choice of operation in patients with pancreatic carcinoma.

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RAPID COMMUNICATION

Genetic changes of *p53*, K-*ras*, and microsatellite instability in gallbladder carcinoma in high-incidence areas of Japan and Hungary

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Abstract

AIM: To disclose geographic differences in genetic changes involved in gallbladder carcinogenesis between two distinct high-incidence areas of Japan and Hungary.

METHODS: We examined 42 cases of gallbladder carcinoma: 22 Japanese and 20 Hungarian cases. *p53* mutations at exons 5 to 8 and K-*ras* mutations at codon 12 were tested by direct sequencing. Microsatellite instability was determined from fluorescent dye-labeled PCR amplifications of five-microsatellite markers (*BAT-25*, *BAT-26*, *D2S123*, *D5S346*, and *D17S250*).

RESULTS: Mutations of *p53* were detected in 11 of 22 Japanese cases and 6 of 18 Hungarian cases (11/22 *vs* 6/18, $P = 0.348$). Transition at CpG sites was found in none of 11 Japanese cases and 2 of 6 Hungarian cases; the difference was marginally significant (0/11 *vs* 2/6, $P = 0.110$). K-*ras* mutations were detected in only one of the Hungarian cases. Eight of 19 (42.1%) Japanese cases were MSI-high (presence of novel peaks in more than one of the five loci analyzed), whereas only 1 of 15 (6.7%) Hungarian cases was MSI-high ($P = 0.047$).

CONCLUSION: It appears that the *p53* mutations and MSI differ in patients with gallbladder carcinoma between two distinct high-incidence areas. Geographic variation might exist in the process of gallbladder carcinogenesis.

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Key words: Gallbladder; Gallbladder Neoplasms; K-*ras*; Microsatellite instability; *p53*

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INTRODUCTION

Although considerable progress has been made regarding the molecular pathogenesis of human neoplasms such as colorectal carcinoma[1,2], pancreatic carcinoma[3,4], and breast carcinoma[5,6], there is only limited information about the genetic changes involved in gallbladder carcinogenesis[7,8]. Gallbladder carcinoma shows striking geographic and ethnic variation[9,10]. The high-incidence areas are scattered throughout the world: Latin America, Eastern Europe, northern India, and Japan[9-13]. The highest mortality rate of gallbladder carcinoma in the world, 35 per 100 000 inhabitants, is found in Southern Chile[9]. Japanese standardized mortality rates for gallbladder carcinoma were world's second highest for males and fifth highest for females in 1996 with a steady increase in incidence (up to 5 per 100 000)[9,10,14]. On the other hand,

Zatonski reported that Hungarian mortality rates of this tumor were the highest in Europe (4 per 100 000 males and 7 per 100 000 females)[13]. This marked geographic variation implies that a combination of genetic and environmental etiologic factors affects the process of carcinogenesis of the gallbladder[10]. Previously, we revealed the geographic variation of gallbladder carcinogenesis between Japan and Chile (a representative of Latin American countries), both of which were known as distinct high-incidence countries, in terms of the *p53* mutational spectra[14]. Only a few other investigators also have reported such geographic and ethnic differences in genetic changes of this tumor[15-17]. Thus, there has been a paucity of evidence regarding the geographic diversity of genetic changes involved in gallbladder carcinogenesis.

The aim of this study was to disclose geographic differences in genetic changes involved in gallbladder carcinogenesis by comparing gallbladder carcinomas from two distinct high-incidence areas, Japan and Hungary (a representative country of Eastern Europe), in terms of *p53* mutations, K-ras mutations, and microsatellite instability (MSI).

MATERIALS AND METHODS

Tissue specimens

From 1982 to 1996, 22 patients with gallbladder carcinoma underwent a resection at Niigata University General Hospital and its affiliated institutions in Niigata Prefecture; the surgical specimens were collected and stored (Japanese cases). All the patients were Japanese. The Japanese cases in this study were identical with the Japanese cases in our previous study[14]. From 1982 to 2001, 20 patients with gallbladder carcinoma underwent a resection in hospitals in Budapest, Hungary; the surgical specimens were collected and stored (Hungarian cases) through the courtesy of a Hungarian oncologist (I.L.) and surgical pathologist (Z.S.). All the patients were residents in Budapest. Both the Japanese cases and the Hungarian cases (a total of 42 cases) formed the basis of this retrospective study. All of the patients gave informed consent for pathologically examining the specimens. No patient in this series was diagnosed with anomalous union of the pancreatic and biliary ducts (AUPBD), and had a family history suggestive of hereditary nonpolyposis colorectal cancer (HNPCC).

The surgical specimens were fixed in formalin and embedded in paraffin. One or two representative sections of each specimen were used for DNA analysis. Histopathological findings were described according to the tumor-node-metastasis (TNM) staging system[18].

DNA preparation

Five serial slices 10-μm thick were cut from each representative histologic section and deparaffinized. Cancer tissue in each slice was dissected under microscopic guidance as described previously[14]. Non-neoplastic tissue in each slice was used as a source of constitutional DNA. DNA was extracted using a DNA Isolator PS Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as described previously[14].

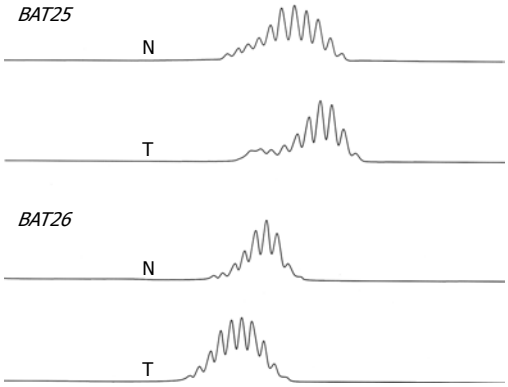


Figure 1 Fragment pattern of case No 1 (Table 1) showing microsatellite instability at two loci (*BAT25*, *BAT26*). N: Normal DNA; T: Tumor DNA.

Analysis of p53 mutations

p53 mutations at exons 5 to 8 were tested by nested polymerase chain reaction (PCR) and direct sequencing as described previously[14].

Analysis of K-ras mutations

K-*ras* mutations at codon 12 were tested by nested PCR, PCR-restriction fragment length polymorphism, and direct sequencing as described elsewhere[19].

Analysis of MSI

Fluorescent dye-labeled PCR amplification was performed using the five microsatellite markers (*BAT-25*, *BAT-26*, *D2S123*, *D5S346*, and *D17S250*) recommended by the National Cancer Institute workshop[20,21]. Fluorescent dye-labeled and unlabeled primers were obtained (Applied Biosystems Japan Ltd., Tokyo, Japan); the 5' oligonucleotide was end-labeled with 6FAM (*BAT-25*, *D2S123*), VIC (*BAT-26*, *D17S250*), or NED (*D5S346*) fluorescent dyes. Amplification by PCR was performed using the Temp Control System PC-700 (ASTEC Co., Ltd., Fukuoka, Japan) in 30-μL reaction volumes containing 100 ng of DNA, 0.75 U of AmpliTaq Gold (Applied Biosystems Japan Ltd.), and 10 pmol of each primer. The cycling profile was: denaturation at 95℃ for 2 min, annealing at 55℃ for 40 s and extension at 72℃ for 40 s followed by a 7-min final extension step. A 1.0-μL aliquot of each fluorescent dye-labeled PCR product was combined with 12 μL of formamide and 0.5 μL of the GENESCAN 350 [ROX] size standard (Applied Biosystems Japan Ltd.) and analyzed on an ABI PRISM 310 Genetic Analyzer using GeneScan Analysis Software (Applied Biosystems Japan Ltd.). MSI experiments were repeated twice to exclude the possibility of a false positive result due to PCR amplification artifacts in an independent PCR reaction. The sequencing results were verified by a second independently generated PCR product.

The presence of MSI was determined from the PCR amplifications of the five-microsatellite markers. Microsatellite instability was defined as the presence of novel peaks that were not found in non-neoplastic tissue (Figure 1). A tumor was defined as having high microsatellite instability (MSI-high) if more than one of

the five loci analyzed showed unequivocal instabilities; a tumor was defined as having low microsatellite instability (MSI-low) if only one locus showed instability; and a tumor was defined as microsatellite stable if no microsatellite instability was found. In this study, MSI-low tumors and microsatellite-stable tumors were categorized together into one group (MSI-low/none), as proposed by recent authors[16,22].

Statistical analysis

Fisher's exact test was used to compare the frequencies of genetic alterations between the two groups. All statistical evaluations were performed using the SPSS 11.5J software package (SPSS Japan Inc., Tokyo, Japan). All tests were two-sided and a P value < 0.05 was considered statistically significant.

RESULTS

p53 mutation

Mutations of *p53* at exons 5 to 8 were detected in 11 of 22 (50.0%) Japanese cases and 6 of 18 (33.3%) Hungarian cases ($P = 0.348$; Table 1). Among the 17 cases with *p53* mutations, transversion was found in 4 of 11 Japanese cases and 1 of 6 Hungarian cases ($P = 0.600$). Transition at CpG sites was found in none of 11 Japanese cases and 2 of 6 Hungarian cases; the difference was marginally significant ($P = 0.110$).

K-ras mutation

K-*ras* mutations at codon 12 were detected in none of 22 Japanese cases and 1 of 20 Hungarian cases ($P = 0.476$) (Table 1).

MSI

Eight of 19 (42.1%) Japanese cases were MSI-high, whereas only one of 15 (6.7%) Hungarian cases was MSI-high; the difference was statistically significant ($P = 0.047$; Table 1).

Association between p53 mutation and MSI in gallbladder carcinoma

Both *p53* mutation and MSI were analyzed successfully in 34 cases: 19 Japanese cases and 15 Hungarian cases (Table 1). When the cases were stratified into Japanese cases and Hungarian cases, *p53* mutations were not associated significantly with MSI in both groups. In all 34 cases, *p53* mutations were associated significantly with MSI (Table 2; $P = 0.033$).

DISCUSSION

Although earlier epidemiologic studies have suggested that the incidence of gallbladder carcinoma varies geographically or ethnically[9,10], there is a paucity of evidence regarding the geographic diversity of molecular changes associated with this tumor[7,8]. This prompted us to conduct the current study. In 1998, we reported that the *p53* mutational spectra of gallbladder carcinoma differed considerably between Japan and Chile, both of which were known as high-incidence countries[14]. The current study first demonstrates that the process of gallbladder carcinogenesis differs in terms of *p53* mutations and microsatellite instability between two distinct high-incidence areas: Japan and Hungary (a representative country of Eastern Europe).

Reported prevalences of *p53* mutation for gallbladder carcinoma ranged from 31% to 70%[14,23-26], suggesting that *p53* mutation plays an important role in the development of this tumor[8]. The prevalences of *p53* mutation in this series (50% in Japanese cases and 33% in Hungarian cases) are comparable with the reported figures. In various malignancies, endogenous carcinogenesis is characterized by transitions at CpG sites, whereas transversions imply the presence of an exogenous mutational process[27-29]. In our previous study, the prevalence of transitions at CpG sites was significantly higher in the Chilean cases than in the Japanese cases[14]. In the current study, there was a trend toward a higher prevalence of transitions at CpG sites in the Hungarian cases. Taken together, these observations suggest that an endogenous mutational process contributed considerably to carcinogenesis of the gallbladder in the Chilean and Hungarian cases. In contrast, considering that there were no transitions at CpG sites and four transversions in the Japanese cases, it appears that exogenous mutations often happen during the process of carcinogenesis in the Japanese cases. Therefore, geographic variation might exist in carcinogenesis of the gallbladder among three distinct areas.

Reported prevalences of K-*ras* mutation for gallbladder carcinoma without AUPBD ranged from 0% to 17%[16,30-33], suggesting that frequency of K-*ras* mutation is relatively low in gallbladder carcinoma without AUPBD. This is consistent with our results: K-*ras* mutation was found in only one of the Hungarian cases. Taken together, most gallbladder carcinoma appears to develop from a K-*ras*-independent pathway.

Microsatellite instability, which represents replication errors, results from DNA mismatches due to environmental or hereditary factors and leads to genomic instability[34,35]. Reported prevalences of MSI-high for sporadic gallbladder carcinoma range from 0% to 10%[15,16,36-38], whereas the prevalence was high (42.1%) in our cases. This suggests that environmental or hereditary factors contribute to carcinogenesis of the gallbladder in some of our cases. In the current study, the prevalence of MSI-high was significantly higher in the Japanese cases than in the Hungarian cases. Considering that there were no patients with HNPCC in this series, the above finding suggests that the high prevalence of MSI-high in the Japanese cases may be due to environmental factors, which remain unknown.

There are inverse relationships between *p53* mutations and MSI in colorectal cancer[39,40]. Regarding gallbladder carcinoma, earlier authors have failed to identify such relationships between *p53* mutations and MSI[16,38]. In this study, *p53* mutations were associated significantly with MSI in all cases. When the cases were stratified into Japanese cases and Hungarian cases, no such associations between *p53* mutation and MSI were found, probably due to the small sample size. Taken together, the above observations suggest that the pathway of carcinogenesis differs between the gallbladder and the colon and rectum.

The current study has limitations. Firstly, it was a

Table 1 Genetic alterations observed in gallbladder carcinomas from Japan and Hungary

Case No	Age (yr)	Sex	Histology[1]		*p53* mutation		K-*ras* mutation	Microsatellite instability
			Type	Grade	Exon/codon	Base change	Base change	
Japanese cases								
1	71	F	PAP	G1	None	-	None	BAT25, BAT26
2	74	F	AD	G1	Exon 5/codon 132	AAG to GAG	None	BAT25, D2S123
3	62	F	PAP	G1	None	-	None	BAT25
4	76	F	AD	G1	Exon 6/codon 193	CAT to AAT	None	BAT25, BAT26
5	78	F	AD	G2	Exon 5/codon 140	ACC to ATC	None	ND
					Exon 5/codon 166	TCA to ACA		
6	70	F	AD	G1	Exon 8/codon 276	GCC to CCC	None	None
7	71	F	AD	G1	None	-	None	BAT26, D2S123
8	79	F	AD	G1	None	-	None	None
9	66	F	PAP	G1	None	-	None	None
10	60	F	PAP	G1	None	-	None	None
11	80	M	PAP	G1	None	-	None	BAT26
12	63	M	PAP	G1	Exon 8/codon 294	GAG to GAA	None	BAT25, BAT26
13	49	F	PAP	G1	None	-	None	D2S123
14	71	M	AD	G2	Exon 8/codon 280	AGA to AAA	None	BAT25, D2S123
15	70	M	AD	G1	Exon 7/codon 238	TGT to CGT	None	ND
16	53	M	AD	G1	None	-	None	None
17	79	F	AD	G3	Exon 8/codon 271	GAG to AAG	None	None
18	62	M	AD	G3	Exon 6/codon 205	TAT to TGT	None	None
19	61	M	AD	G1	None	-	None	D17S250, D2S123
20	69	M	AD	G2	Exon 7/codon 231	ACC to ATC	None	BAT25, D17S250
21	58	F	AD	G2	Exon 5/codon 160	ATG to GTG	None	ND
					Exon 6/codon 220	TAT to AAT		
22	76	F	AD	G3	None	-	None	None
Hungarian cases								
23	71	M	AD	G2	Exon 7/codon 249	AGG to ACG	None	BAT26
24	69	F	AD	G3	Exon 6/codon 213	CAG to CGG	None	None
25	69	F	AD	G2	None	-	None	None
26	76	F	AD	G2	None	-	None	None
27	61	F	AD	G3	None	-	None	None
28	58	F	AD	G3	None	-	None	BAT25
29	69	F	AD	G3	None	-	None	None
30	75	F	PAP	G1	None	-	None	None
31	66	F	AD	G3	None	-	None	None
32	65	F	AD	G1	Exon 6/codon 219	CCC to TCC	None	ND
33	61	F	AD	G3	ND	-	None	ND
34	Unknown	F	PAP	G1	Exon 5/codon 138	GCC to GCT	None	BAT25, D17S250
					Exon 5/codon 167	CAG to CAA		
					Exon 5/codon 170	ACG to ACA		
35	Unknown	F	PAP	G1	Exon 5/codon 158	CGC to TGC	GGT to AGT	ND
					Exon 6/codon 223	CCT to TCT		
36	Unknown	F	AD	G3	Exon 6/codon 210	AAC to AAT	None	ND
					Exon 7/codon 228	GAC to AAC		
37	Unknown	F	AD	G2	ND	-	None	ND
38	53	F	AD	G3	None	-	None	None
39	72	Unknown	AD	G1	None	-	None	None
40	69	M	AD	G1	None	-	None	BAT26
41	57	F	AD	G1	None	-	None	None
42	74	F	AD	G1	None	-	None	BAT25

[1]According to the tumor-node-metastasis (TNM) staging system[18]. PAP: Papillary carcinoma; AD: Adenocarcinoma; ND: Not detected; G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated.

Table 2 Association between *p53* mutation and MSI in gallbladder carcinoma

		MSI-high	MSI-low/none	*P*
p53 mutation	Present	6	5	0.033
	Absent	3	20	

MSI: Microsatellite instability.

retrospective analysis of a small number of patients. Secondly, DNA preparation from paraffin-embedded tissue sections was unsuccessful in some patients. Thirdly, only limited clinical information was available in the Hungarian cases to protect the patients' privacy. To our knowledge, however, this study demonstrates more clearly the geographic diversity of gallbladder carcinogenesis than earlier reports[15-17].

In conclusion, it appears that the *p53* mutations and MSI differ in patients with gallbladder carcinoma between two distinct high-incidence areas. Exogenous mutations

and unknown environmental factors may play roles in gallbladder carcinogenesis in the Japanese cases, whereas the Hungarian cases are characterized by an endogenous mutational process. Thus, geographic variation might exist in the process of gallbladder carcinogenesis.

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COMMENTS

Background

Although considerable progress has been made regarding the molecular pathogenesis of human neoplasms, there is only limited information about the genetic changes involved in gallbladder carcinogenesis. Gallbladder carcinoma shows striking geographic and ethnic variation, however there is a paucity of evidence regarding the geographic diversity of molecular changes associated with this tumor.

Research frontiers

In 1998, we reported that the *p53* mutational spectra of gallbladder carcinoma differed considerably between Japan and Chile, both of which were known as high-incidence countries. Only a few other investigators also have reported such geographic and ethnic differences in genetic changes of this tumor. The aim of this study was to disclose geographic differences in genetic changes involved in gallbladder carcinogenesis by comparing gallbladder carcinomas from two distinct high-incidence areas, Japan and Hungary.

Innovations and breakthroughs

The current study first demonstrates that the *p53* mutations and MSI differ in patients with gallbladder carcinoma between two distinct high-incidence areas: Japan and Hungary. Exogenous mutations and unknown environmental factors may play roles in gallbladder carcinogenesis in the Japanese cases, whereas the Hungarian cases are characterized by an endogenous mutational process. Thus, geographic variation might exist in the process of gallbladder carcinogenesis.

Applications

Gallbladder carcinoma is a highly lethal disease with a poor prognosis. Therefore, it would be very beneficial to identify the molecular mechanism responsible for this condition. Advances in the understanding of the genetic changes of this tumor will help in understanding the pathogenesis of this miserable disease.

Terminology

Microsatellite instability (MSI) is caused by a failure of the DNA mismatch repair system to repair errors that occur during the replication of DNA and is characterized by the accelerated accumulation of single nucleotide mutations and alterations in the length of simple, repetitive microsatellite sequences that occur ubiquitously throughout the genome. MSI is seen in most HNPCC tumors and proportion of nonhereditary colorectal tumors. The presence of MSI in tumor tissue is associated with certain unique clinical and pathological characteristics.

Peer review

This study aimed to determine the difference of some genetic changes involved in gallbladder carcinogenesis in high-prevalence areas of Japan and Hungary. This is a well written paper and has a nice finding. While it is simply an observational study, I think it is an important observation.

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RAPID COMMUNICATION

Midkine secretion protects Hep3B cells from cadmium induced cellular damage

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Abstract

AIM: To evaluate role of midkine secretion during Cadmium (Cd) exposure in the human hepatocyte cell line Hep3B cells.

METHODS: Different dosages of Cd (0.5-1-5-10 μg/mL) were applied to Hep3B cells and their effects to apoptosis, lactate dehydrogenase (LDH) leakage and midkine secretion were evaluated as time dependent manner. Same experiments were repeated with exogenously applied midkine (250-5000 pg/mL) and/or 5 μg/mL Cd.

RESULTS: Cd exposure induced prominent apoptosis and LDH leakage beginning from lower dosages at the 48th h. Cd induced midkine secretion with higher dosages (P < 0.001), (control, Cd 0.5-1-5-10 μg/mL respectively: 1123 ± 73, 1157 ± 63, 1242 ± 90, 1886 ± 175, 1712 ± 166 pg/mL). Exogenous 500-5000 pg/mL midkine application during 5 μg/mL Cd toxicity prevented caspase-3 activation (control, Cd toxicity, 250, 500, 1000, 2500, 5000 pg/mL midkine+ Cd toxicity, respectively: 374 ± 64, 1786 ± 156, 1545 ± 179, 1203 ± 113, 974 ± 116, 646 ± 56, 556 ± 63 cfu) LDH leakage and cell death in Hep3B cells (P < 0.001).

CONCLUSION: Our results showed that midkine secretion from Hep3B cells during Cd exposure protects liver cells from Cd induced cellular damage. Midkine has anti-apoptotic and cytoprotective role during Cd toxicity. Further studies are needed to explain the mechanism of midkine secretion and cytoprotective role of midkine during Cd exposure. Midkine may be a promising therapeutic agent in different toxic hepatic diseases.

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Key words: Cadmium; Midkine; Hepatocyte; Apoptosis; Caspase-3; Lactate dehydrogenase

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INTRODUCTION

Cadmium (Cd) exposure occurs widely in the general population, especially low-level chronic exposure through smoking and dietary sources, but it is known as one of the most toxic environmental and industrial pollutants. Cd accumulates in the body because of slow excretion[1]. Cd causes toxicity in different organs. Acute and chronic Cd exposure mostly results in hepatotoxicity[2]. It seems that the level of damage depends on the dosage and duration of Cd application. Exposure of cells to toxic chemicals is known to up-regulate the expression of a number of stress proteins and results in activation of apoptotic pathways and consequently cellular damage. *In vivo* and *in vitro* studies showed that inflammation and oxidative damage are main mechanisms of Cd induced toxicity[3-6]. Cd induces mitochondria-dependent apoptotic pathways where caspase-3 and caspase-9 are activated[7].

Midkine is a heparin binding growth factor. It takes part in cancer and inflammation[8]. Although midkine is a mitogenic factor during carcinogenesis, it plays a critical role in ischemia induced inflammatory damage[9]. It was demonstrated that midkine acts as an antiapoptotic factor in HepG2 cells; furthermore, midkine suppressed the activity of caspase-3, which plays a significant role in the apoptotic pathway. Pretreatment with midkine prevents tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis in the HepG2 cells[10]. TRAIL alone triggered massive apoptosis accompanied by caspase activation in tissue explants from patients with liver steatosis or hepatitis C viral

infection[11]. TNF-alpha, released from nonparenchymal cells as well as associated cytokines, are responsible for clinical expression and tissue damage observed with cadmium-induced hepatotoxicity[12]. It was shown that midkine expression was upregulated in a marine gastropod limpet patella caerulea after they were exposed to sublethal doses of Cd[13]. But, whether midkine takes part in Cd induced mechanisms in human cells is still unknown. In this study we aimed to evaluate effects of Cd induced midkine secretion in the human hepatocyte cell line Hep3B cells, and its effects to cellular proliferation, apoptosis and biochemical parameter of cellular integrity during Cd exposure.

MATERIALS AND METHODS

Cell lines, chemicals and materials

Human hepatoma cell line Hep3B cells were obtained from the ATCC. Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (PAA, Austuria), supplemented with fetal calf serum (FCS), (PAA, Austuria), L-glutamine (Sigma, USA), streptomycin (Sigma, USA) and penicillin (Sigma, USA). Cadmium ($CdCl_2$, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in water, sterilized by 0.22 μm pore size cellulose acetate membrane filters, and added to cultures at the indicated time and concentrations. $CdCl_2$ toxicity was studied in Hep3B cell line. Human recombinant midkine was obtained from Peprotech (UK). Cell counts were tested by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, Sigma, USA). For evaluation of apoptosis caspase-3 levels were measured by a flourometric kit (Biotium, USA). Lactate Dehydrogenase (LDH) level was measured with a kit using an automatic multianalyzer (Roche; P800). Midkine levels were measured by an ELISA development kit (Peprotech,UK).

Cell culture and experimental protocol

The human hepatoma cell line Hep3B was cultured in RPMI-1640 medium, supplemented with 10% v/v fetal calf serum, 2 mmol/L L-glutamine, streptomycin (100 μg/mL) and penicillin (100 IU/mL) in a humidified atmosphere containing 5% CO_2 at 37℃. One day before the experiments, cells were seeded on 96-well microtitre plates (Nunc, Denmark) at 2×10^5 cells/mL.

Depending on the groups, different concentrations of cadmium (0.5-1-5-10 μg/mL) and midkine (250, 500, 1000, 2500, 5000 pg/mL) were added to medium for 48 h. For evaluation of the effects of midkine during cadmium toxicity; cadmium was used at a dosage of 5 μg/mL. $n = 6$, for every experimental group.

LDH and caspase-3 levels were evaluated from cadmium and/or midkine treated cells at the 48th h. MTT was measured at the 2nd, 24th and 48th h. Midkine was measured from supernatants. After supernatants were removed cell surface was washed with sterile phosphate buffered saline (PBS) and cells were harvested with lysis solution and caspase-3 levels of groups were measured from cell lysates. LDH measurement was done from both of the supernatant and cell lysates.

Evaluation of cellular proliferation or death

MTT, a colorimetric assay based upon the ability of living cells to reduce MTT into formazan, was used for evaluation of the effects of dose and time dependent effects of cadmium and midkine on cellular death or proliferation (2nd, 24th, 48th h). Cell number % was calculated as ratio of cell number of effected group *vs* control group × 100 at the determined hour.

Biochemical determination of cell death

Hep3B cells were plated in 96 multiwell cell culture plates as 3×10^5 cells/mL. LDH is normally present in the cytosol of hepatocytes. In response to cell damage LDH is released from the cells. Therefore, to determine cell death, we measured secreted and intracellular LDH levels and calculated % released LDH at the 48th h for each group. To do this, the medium was collected to measure enzyme activities. The adherent cells were lysed. Both medium and cell lysates were used for quantitative determination of LDH activity (IU/L) which was performed with an automatic multianalyzer (Roche) using a kit (Roche). Released enzyme fractions for each sample were calculated as the ratio of enzyme present in the medium *vs* the sum of the levels of same enzyme in the supernatant and in the cells.

Measurement of apoptosis

Caspase-3 levels: The presence of apoptosis was determined by caspase-3 levels. Equal numbers of cells were used for caspase-3 level measurements. Cells were lysed with assay buffer (50 mmol/L HEPES, pH 7.4, 100 mmol/L NaCl, 0.1% CHAPS, 10 mmol/LM DTT, 2 mmol/L EDTA, 2 mmol/L EGTA, Triton X-100, 0.1%). Caspase-3 levels were measured by DEVD-R110 Fluorometric HTS Assay Kit from cell lysates. The fluorogenic substrate $(Ac\text{-}DEVD)_2$-R110 was used for this assay. It is completely hydrolyzed by the enzyme in two successive steps. Cleavage of the first DEVD peptide results in the monopeptide Ac-DEVD-R110 intermediate, which has absorption and emission wavelengths similar to those of R110 ($\lambda_{abs}/\lambda_{abs}$ = 496/520 nm), but has only about 10% the fluorescence of the latter. Hydrolysis of the second DEVD peptide releases the dye R110, leading to a substantial fluorescence increase.

Equal volumes of sample and caspase-3 detection buffer were added to assay plate, and then incubated at 37℃ for 1 h in an incubator. Results were read with a fluorometer at 470 nm excitation filter and 520 nm emission filters. R110 was used for generating a standard curve to calculate amount of substrate conversion.

Statistical analysis

Results of the experiments were analyzed by One Way ANOVA, followed by a multiple comparison test using SPSS 10.0. $P < 0.05$ was accepted as statistically significant. Results were given as mean ± SEM.

RESULTS

Cell proliferation and toxicity

We characterized the concentration-dependent cytotoxic effect of Cd on human hepatocyte cell line as a function of time. Cadmium exposure decreased living cell number depending on the dosage. Minimal cytotoxic (< 5%) effect was seen at the 0.5 μg/mL dosage. As shown in Figure 1,

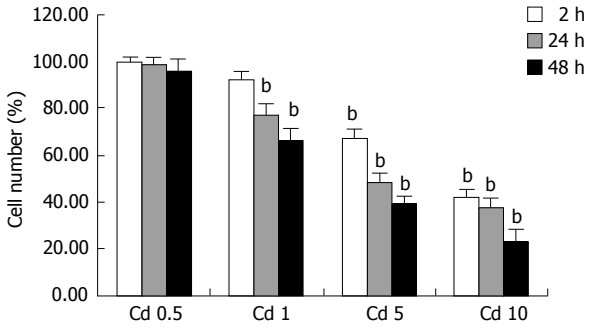


Figure 1 Cell death (%) was determined at 2nd, 24th and 48th h by the MTT assay. Cd exposure induced prominent cell death in Hep3B hepatocytes with dose and time dependent manner. Data are from 6 independent experiments for each condition. Data are presented as mean ± SEM. $^bP < 0.001$ *vs* control group.

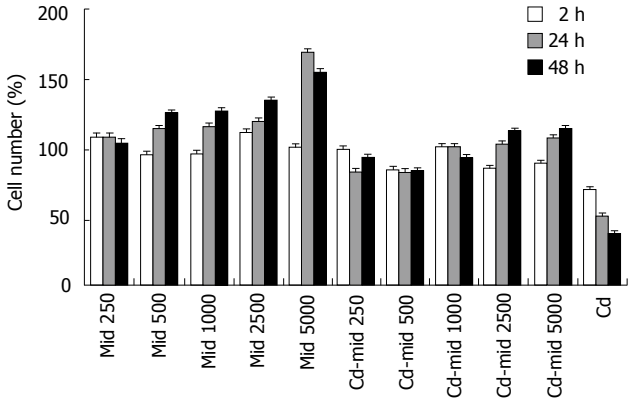


Figure 2 Cell number (%) was determined by MTT assay following 2nd, 24th and 48th h exposure to 250-5000 pg/mL and/or 5 μg/mL Cd. Midkine treatment increased cell proliferation in Hep3B hepatocytes with dose and time dependent manner. Data are presented as mean ± SEM.

cell exposure to 1 μg/mL Cd for up to 2 h only slightly affected cell viability as revealed by MTT measurements compared to control values estimated in untreated cells, but it becomes apparent at the 24th and 48th h. Cd exposure caused cellular damage in a dose and time dependent manner in the Hep3B cell line. Cytotoxicity was more prominent with higher dosages at the 24th and 48th h ($P < 0.001$, Figure 1). Regarding to these data, 5 μg/mL $CdCl_2$ concentration which with moderate-high toxic impact was chosen for subsequent experiments with different dosages of midkine.

Midkine treatment caused proliferation of Hep3B cells in a dose and time dependent manner compared to control group. The highest increase in cell number was at the 48th h and 5000 pg/mL midkine concentration ($P < 0.001$, Figure 2). Midkine treatment during Cd toxicity prevents cell death, even with the lowest dosages ($P < 0.001$, Figure 2).

Determination of apoptosis

Increased apoptosis was seen in Cd treated cells, which was confirmed with increased caspase-3 levels. Lowest dosage of Cd application did not increase caspase-3 levels compared to untreated cells. Activation of caspase-3 started at the 1 μg/mL Cd dosage ($P < 0.001$, Figure 3).

Midkine treatment decreased caspase-3 levels in the

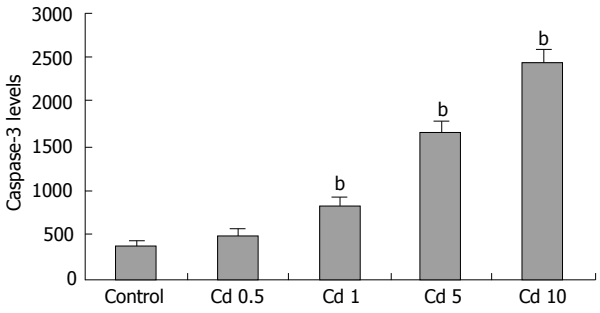


Figure 3 Caspase-3 levels were measured following a 48 h exposure to 0.5-10 μg/mL Cd. Data were given as mean ± SEM. $^bP < 0.001$ *vs* control group.

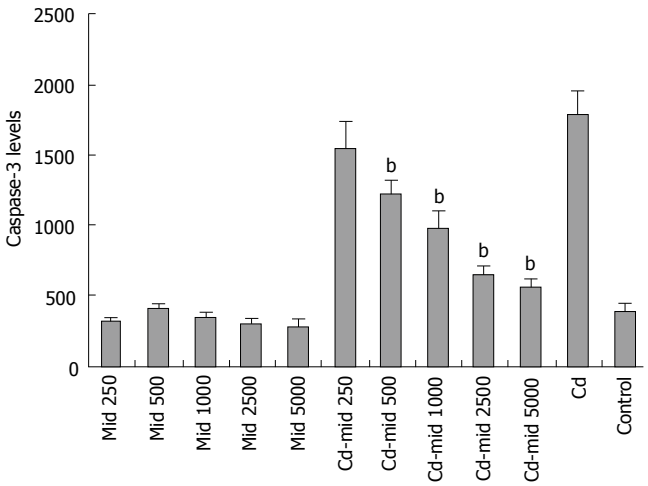


Figure 4 Caspase-3 levels were measured following a 48 h of exposure to 250-5000 pg/mL Midkine and/or 5 μg/mL Cd. Data are presented as mean ± SEM. $^bP < 0.001$ *vs* control group.

Hep3B cells. It prevents Cd induced apoptosis prominently starting from 500 pg/mL concentration of midkine application ($P < 0.001$, Figure 4).

Cytotoxic effects of Cd in the hepatocytes

Incubation of Hep3B cells with Cd resulted in cytotoxicity as assessed by LDH released into the incubation media. LDH release in the Hep3B cells to media started at the 1 μg/mL Cd dosage (Figure 5).

Midkine treatment at the same time with 5 μg/mL Cd exposure decreased LDH release in the Hep3B cells (Figure 6).

Midkine secretion

Measurable basal midkine secretion was found in the Hep3B cells under normal conditions. Cd treatment induced midkine secretion in the Hep3B cells in a dose dependent manner (Figure 7).

DISCUSSION

Acute/chronic Cd exposure mostly results in hepatotoxicity, where it is a good model to study toxic substance-induced liver damage[2]. Midkine family has strong anti-apoptotic function so they are obviously considered non specific (i.e., for Cd) mechanisms of defence[10]. Intense midkine expression has also been found in increased various human tumors and level of midkine expression correlates

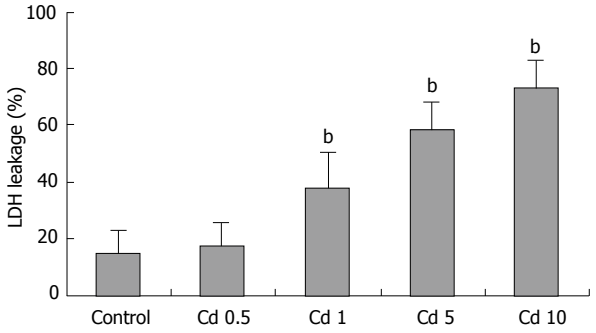


Figure 5 Cd induced cytotoxicity at the 48th h of experiment determined by % LDH released to medium. Starting from the 1 μg/mL dosage Cd treatment caused prominent LDH release from hepatocytes at the end of 48th h ($P < 0.001$). Data are presented as mean ± SEM. $^bP < 0.001$ *vs* control group.

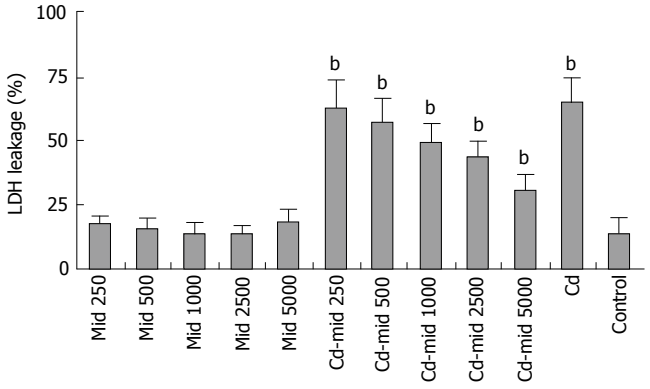


Figure 6 Effects of 48 h midkine (250-5000 pg/mL) and/or 5 μg/mL Cd treatment on the LDH leakage in the Hep3B cells. Data are presented as mean ± SEM. $^bP < 0.001$ *vs* control group.

negatively with the patients' prognosis[14,15]. Furthermore, midkine accumulation is noted in senile plaques in the brains of Alzheimer's disease patients[16]. Midkine is expressed around the damaged neuronal site after cerebral infarction[17], suggesting a role for midkine in tissue repair.

The results of the present study supports the idea that Cd exposure causes cytotoxicity and apoptosis in the Hep3B cells. In both time and dose-response studies, LDH leakage, which is very important parameter to detect hepatocellular integrity, was greater in Cd treated cells. These effects were more prominent at the 48th h. During Cd exposure, activation of caspase-3 was detected in Hep3B cells, suggesting a caspase-dependent pathway is involved in Cd toxicity. Cd can upregulate the expression of a number of genes that produce products that can detoxify Cd and/or repair Cd induced lesions. Our studies showed that midkine is one of them. The induction pathways or receptors of midkine expressed by Cd exposure is still unclear. Midkine is multifunctional heparin-binding growth factor and cytokine and has anti-apoptotic and cell-protecting activities[8]. Untreated Hep3B cells have also a basal midkine secretion. In our study, midkine treatment decreased apoptosis and increased cellular proliferation in a dose and time dependent manner. It has cytoprotective, anti-apoptotic effects against Cd toxicity in Hep3B cells. It seems that midkine is produced endogenously and released in to medium as a defense mechanism

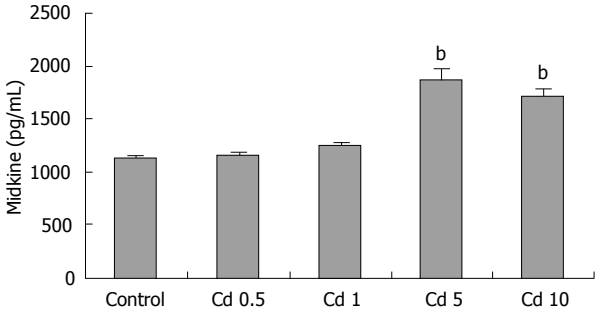


Figure 7 Effects of 0.5-10 μg/mL Cd treatment on midkine secretion in the Hep3B cells. With 0.5 and 1 μg/mL Cd exposure we obtained similar midkine secretion as untreated cells. Midkine secretion was highest as a response to 5 μg/mL Cd treatment dosage. Data are presented as mean ± SEM. $^bP < 0.001$ *vs* control group.

of Hep3B cells against Cd toxicity. Among midkine receptors, receptor-type protein tyrosine phophatase z (PTP z) has been studied extensively. Midkine stimulates phosphorylation of specific members of the JAK/STAT pathway, namely JAK1, JAK2, and STAT1α [18,19]. In addition, low density lipoprotein receptor-related protein (LRP) has also been identified as a receptor[20]. The midkine receptor is considered to be a molecular complex containing these proteins. The downstream signaling systems of these receptors include ERK, which participates in the reduction of necrotic and apoptotic cell death[21]. Internalization of midkine in to cell and nuclear targeting is important for its antiapoptotic function[22]. Activation of these receptors and intracellular pathways might take part in cytoprotective effects of midkine during Cd toxicity. Human and experimental studies have shown that apoptosis plays a role in hepatocyte death in alcoholic liver disease and nonalcoholic steatohepatitis and apoptosis levels correlate with the severity of the liver disease[23-26]. LDL receptor-related protein (LRP) is another midkine receptor. LRP is important for lipid and lipoprotein uptake to cells[27]. Lipid profiles of steatohepatitis patients were found disturbed[28]. It was shown that midkine takes part in the inflammatory and repair processes after partial hepatectomy. They suggested that midkine is beneficial for liver regeneration[29]. ERK, JNK signal pathways disturbed during Cd toxicity are activated by midkine[18,19,22,30]. Beneficial effects of exogenous midkine to minimize Cd induced damage would provide a new perspective for innovation in the treatment of Cd intoxications and in NonAlcoholic Fatty Liver Disease (disease near exclusively characterized by apoptotic process), in Drug Induced Liver Injury and in the combined form, illnesses far long more evident than Cd intoxication in the every day practice of gastroenterologists and hospitals. But further acute and chronic *in vitro* and *in vivo* studies are needed to evaluate which intracellular pathway(s) is activated during these processes.

COMMENTS

Background

Acute/chronic Cadmium (Cd) exposure mostly results in hepatotoxicity, where it is a good model to study toxic substance-induced liver damage. Midkine is expressed around the damaged tissues after ischemic damage and suggesting a role for midkine in tissue repair. Its expression has been found in tumors and level

of midkine expression correlates negatively with the patients' prognosis. Midkine is cytoprotective and has anti-apoptotic effect.

Research frontiers

Beneficial effects of exogenous midkine to minimize Cd induced damage would provide a new perspective for innovation in the treatment of Cd intoxications and other liver diseases.

Innovations and breakthroughs

Cd is well known environmental toxic substance which mainly damages liver. In this study we showed that Cd treatment induces midkine secretion from hepatocytes. Midkine might have protective role during Cd toxicity.

Applications

These finding may be used in the different liver diseases such as alcoholic, toxic and non- alcoholic liver disease models.

Peer review

In this experimental *in vitro* study, the authors showed that midkine secretion from Hep3B cells during Cd exposure protects liver cells from Cd induced cellular damage. Midkine has anti-apoptotic and cytoprotective role during Cd toxicity. Midkine may be a promising therapeutic agent in different toxic hepatic diseases.

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RAPID COMMUNICATION

Effects of primary suture and fib sealant on hemostasis and liver regeneration in an experimental liver injury

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Abstract

AIM: To investigate the effects of fibrin sealant on hemostasis and liver regeneration and intra-abdominal adhesions in an experimental liver injury.

METHODS: Thirty-six Wistar rats were randomly divided into primary suture group (n = 15), fibrin sealant group (n = 15) and control group (n = 6). A wedge resection was performed on the left lobe of the liver. In primary suture group, liver was sutured using polypropylene material, while fibrin glue was administrated on the liver surface in fibrin sealant group.

RESULTS: More intra-abdominal adhesions were observed in the primary suture group compared to the fibrin sealant group on 3rd (2.50 ± 0.5 *vs* 0.25 ± 0.5, P = 0.015), 10th (2.75 ± 0.5 *vs* 0.50 ± 0.6, P = 0.06) and 20th (1.75 ± 0.5 *vs* 0.70 ± 0.5, P = 0.015) postoperative days. Histopathological scores were better in the fibrin sealant group in comparison with the primary suture group on 3rd (8.75 ± 0.5 *vs* 6.75 ± 0.5, P = 0.006), 10th (7.50 ± 1.0 *vs* 5.5 ± 0.6, P = 0.021) and 20th (6.40 ± 1.7 *vs* 3.20 ± 1.6, P = 0.025) postoperative days.

CONCLUSION: Out data suggest that fibrin sealant is preferred over primary suture in appropriate cases including liver trauma since it causes less intra-abdominal adhesions while allowing shorter hemostasis time as assessed in experimental liver trauma.

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Key words: Liver; Trauma; Fibrin sealant; Hemostasis; Regeneration

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INTRODUCTION

Liver injuries occur as a result of blunt and penetrating traumas and rarely due to some iatrogenic reasons. In blunt abdominal traumas, liver turns out to be the organ that mostly gets injured, with mortality rates varying from 10% to 15%. Eighty percent of the liver injuries are formed subsequent to penetrating wounds caused by fire guns or piercing, incisive tools. Acute bleedings and operative complications cause an increase in mortality and morbidity rates[1]. Fibrin glue has been used in a wide selection of surgical fields, such as thorax surgery[2-4], and otolaryngology[5], and neurosurgery[6], and cardiovascular surgery[7]. Besides this, in general surgery application of fibrin glue has been made for treatment[8,9] of anal fistulas and repair of inguinal hernia. Fibrin sealant can be used as a safe and appropriate treatment technique in liver injuries. However, its effects on liver regeneration and development of intra-abdominal adhesions yet remain unclear. In this experimental study, the early and late effects of the reparation techniques employing primary sutures and fibrin glues on an experimental trauma model are evaluated.

MATERIALS AND METHODS

The study was performed at the animal laboratory of Ministry of Health Ankara Training and Research Hospital, after obtaining an approval from the Ethics Committee. For the purposes of the study, 36 male albino Wistar rats were selected from ages varying between 18 to 20 wk and an average body weight of 160 g. Animals were fed standard rodent food and water. They were left hungry for 24 h during both the pre- and post-operative periods. Animals did not receive antibiotic prophylaxis. The experimental study was conducted under semi-sterile conditions.

Rats were randomly divided into three groups: (1) Primary suture group consisting of 15 rats; (2) fibrin sealant group consisting of 15 rats; and (3) control group (which was used only for bleeding time measurements) consisting of 6 rats. Anesthesia was induced with 75 mg/kg ketamin HCl and 5 mg/kg xylazine HCl (Rompun®)

Table 1 Findings obtained from experimental groups

Subgroup	Primary suture			FIbrin sealant			Control
	A	B	C	A	B	C	
Adhesion score	2.50 ± 0.5	2.75 ± 0.5	1.75 ± 0.5	0.25 ± 0.5 ($P < 0.015$)	0.50 ± 0.6 ($P < 0.06$)	0.70 ± 0.5 ($P < 0.015$)	-
Histopathologic score	8.75 ± 0.5	7.50 ± 1.0	6.40 ± 1.7	6.75 ± 0.5 ($P < 0.006$)	5.50 ± 0.6 ($P < 0.021$)	3.20 ± 1.6 ($P < 0.025$)	-
Bleeding time(s)		113 ± 78			59 ± 37 ([a]$P < 0.001$)		125.7 ± 74

A: Postoperative d 3; B: Postoperative d 10; C: Postoperative d 20. [a]Mann-Whitney *U* test, according to the control group.

im. Then the abdominal wall was shaved off all the hair and the skin was cleaned using Betadine® solution. The abdomen was then entered with a 3-cm median incision to resect a triangular piece with a side length of 1 cm from the inferior edge of the left liver lobe (approximately 4% of the overall liver weight). In the primary suture group, horizontal matrix sutures (6/0 polypropylene) were applied to repair the liver injury. In the fibrin sealant group, the liver surface was coated with fibrin sealant (Tissel Kit®, 2.0 mL, Baxter AG). The fibrin sealant kit contains proteins and thrombin, properly cooled and dried, as well as a $CaCl_2$ and aprotinin solution. Mixing these substances results in two components: a covering and a thrombin solution. After the administration of primary suture and fibrin sealant, both test groups underwent chronometric measurements to determine the bleeding times. Blood samples (1 mL) were taken from the inferior vena cava to detect AST, ALT and ALP values. Five rats from each of the two test groups were sacrificed by administration of ether anesthesia at high doses on 3rd, 10th and 20th postoperative days. Laparotomy was performed by an U-shaped incision curved to upwards. Development of intra-abdominal adhesions was then assessed in animals for qualitative aspects, which were defined formerly as the following phases: Phase 1: Avascular, transparent, thin adhesion; Phase 2: Partly vascular, medium thick adhesion distinguishable with blunt dissection; Phase 3: Vascular, barely thick adhesion distinguishable with sharp dissection[10]. Samples were taken from the recovering liver region.

Light microscopy was employed for the evaluation and scoring of hepatic regeneration based on the following criteria: 1 = necrosis, 2 = hemorrhage, 3 = cytoplasmic vacuolization, 4 = multinuclear large cells, 5 = fibro-vascular structures, 6 = inflammatory exudates. Scores ranging from 0 to 3 were interpreted as the following: 0 = absence of any of these parameters, 1 = slight levels, 2 = medium levels, and 3 = high levels attained by values for the same, which were processed for calculating the total histopathologic regeneration score[11]. Points calculated at high levels indicated that the regeneration had an immature character. Total histopathologic regeneration scores were calculated for each group. AST, ALT and ALP values were assessed as the liver enzymes indicating cellular damage.

Mann-Whitney *U* and Chi-square tests were used for statistical analyses when appropriate.

RESULTS

The mean bleeding times were 125.7 ± 74 s, 113 ± 78 s and 59 ± 37 s in the control group, primary suture group and fibrin sealant group, respectively, indicating that the bleeding time was significantly shorter in the fibrin sealant group as compared to the control group and primary suture group ($P = 0.001$). However, no significant difference ($P = 0.069$) in bleeding time was found between the control group and primary suture group. Results of the test groups are shown in Table 1.

In the primary suture group, tearing on the liver surface and bleeding were observed during the suturing procedure; abscess formation was noted on suture lines over the liver in one rat on d 3, in an another rat on d 10 and in two other rats on d 20 postoperatively. At the 10th and 20th postoperative days, wound regions could not be observed clearly due to intensive adhesions between the liver and the great omentum.

A second administration of fibrin sealant was required in two rats of the fibrin sealant group due to the separation of the wound surfaces on d 3. In this group, the sites of hepatic resection were proven to be indistinguishable from the normal parenchyma on the 20th postoperative day; and there was no abscess development in the any rat liver throughout the postoperative study period; few adhesions were observed between the liver and the omentum. Although the AST, ALT and ALP levels were found to be lower in fibrin sealant group on the 3rd, 10th and 20th as compared to the primary suture group, but the difference could not reach statistical significance.

Significantly more intra-abdominal adhesions were observed in the primary suture group compared to the fibrin sealant group on 3rd ($P = 0.015$), 10th ($P = 0.006$) and 20th ($P = 0.015$) postoperative days.

The mean histopathological regeneration scores indicated a significantly more wound immaturity in the primary suture group than in the fibrin sealant group on the 3rd ($P = 0.006$), 10th ($P = 0.021$) and 20th ($P = 0.025$) postoperative days. In the liver regeneration areas of necrosis and hemorrhage appeared to be less in the fibrin sealant group on the 3rd postoperative day ($P = 0.014$ and $P = 0.003$, respectively) as compared to the primary suture group. Cytoplasmic vacuolization was found to be significantly higher in primary suture group on the 20th postoperative day ($P = 0.014$) as compared to the primary suture group.

DISCUSSION

Deep hepatic sutures are known to prove inefficient in

stopping bleeding from the portal veins and branches of hepatic arteries as well as the posterior hepatic vein[12]. Recent studies tend to concentrate on investigating the risk of formation of intra-hepatic hematoma and abscess as well as the development of areas of necrosis dependent on parenchymal ischemia[12]. In the present study, the parenchymal suture group required a significantly longer duration for hemostasis (113 ± 78 s) than the fibrin sealant group (59 ± 37 s). We prefer using polypropylene sutures, a synthetic non-absorbable material known to have a more inert composition and pose lesser risk for infections. In some previous studies, it has been demonstrated that the thrombus generated after the administration of fibrin sealant may have less potential for the occurrence of the infection[13].

In the present study, the fibrin sealant provided shorter hemostasis times following the application. It was hard to re-find the operation area in fibrin sealant administered rats even on the 3rd postoperative day. In two rats, however, an extra fibrin sealant application was required to ensure the merging of the two surfaces, yet neither of these rats indicated any signs of secondary bleeding on surfaces of injured livers. Jacob *et al*[14] have recently reported that fibrin sealants are beneficial even in cases with prolonged hemostasis in rats with liver injuries. They demonstrated that fibrin impregnated collagens-enhanced survival compared to primary sutures, fibrin sealant was absorbed completely on d 28. In another study[15] performed on dogs, an efficient hemostasis was obtained using fibrin sealant in both superficial and profound injuries, no signs of hematoma or secondary bleeding were encountered and the fibrin sealant was completely absorbed within 6 wk of application. Chonn *et al*[16] administered the fibrin sealant in addition to performing surgery using standard surgical techniques subsequent to a liver injury created by infliction of an external blast effect. However, most of subjects in the control group required perihepatic packing, in the fibrin sealant administered group none of the subjects revealed a need for perihepatic packing and re-laparatomy[16]. Similarly, another study demonstrated that fibrin glue eliminated the need for packing after severe liver injuries[17]. Holcomb *et al*[18] attempted to apply the dry fibrin sealing dressing composed of concentrated fibrinogen, thrombin and calcium on polyglyconate mesh on pigs with grade V experimental liver injury. They consequently reported that dry fibrin sealing dressing provided a simple but quick and efficient control over hemorrhage without change of efficacy with neither hypothermia nor coagulopathy[18]. Moreover, several experimental studies have advocated the effectiveness of the novel fibrin sealant[19-21].

In this study, the highest incidence of abscess formation (33%) was observed in the primary suture group postoperatively, while no abscess was observed in the fibrin sealant group. This can be explained by the features of this technique which does not allow occurrence of any ischemic or blind areas or hematoma formation, and the fibrin sealant generates a quick and permanent sealing on blood and lymphatic veins by stopping fibrin exudation. According to the Dulchavsky's study, autologous fibrin gel possesses bactericidal properties in contaminated hepatic injuries[22]. Likewise, in the Taha's experimental study, the number of occurrences of abscesses was less extensive in the fibrin adhesive group than the suture group[23].

Dulchavsky's study with fibrin gel has demonstrated a significant improvement in adhesion formation and intra-abdominal abscess rate as compared with suture hepatorrhaphy[22]. The grade of adhesions found between the liver and omentum was observed to be drastically lower in the fibrin sealant group, the reason of which was attributed to minimal tissue damage and non-existent ischemia in liver parenchyma ensured with this technique[22].

The results obtained in this study are in compliance with those of past studies showing completion of liver, spleen and renal regeneration on d 30 following fibrin sealant administration[11]. Wound healing appeared to be completed at a high extent based on the findings of macroscopic evaluations in this group on d 20. The histological findings obtained also showed that hepatic healing was at higher rates in the fibrin sealant group than in the other groups. Tovar *et al*[11] investigated the effects of fibrin sealant administration on hemostasis and hepatic healing after hepatectomy. Fibrin sealant administration technique provided a faster hemostasis, while the hepatic recovery in the same group revealed to be quicker compared to hot air coagulation and primary suture techniques[11]. The results of this study are in agreement with those obtained in our experimental study. Previous studies demonstrated that plasminogen activators had an important additive role in liver regeneration by their contribution on remodelling of the liver[24,25]. Contrary to the presence of aprotinin in the fibrin sealant kit which is known as an plasminogen inhibitor, no negative effect on hepatic healing was observed. Vice versa, hepatic healing was more rapid than the other group. We believe that further studies need to be performed on this particular issue.

Kohno *et al*[26] compared the efficiencies of microcrystal collagen dust versus the fibrin sealant following an elective hepatic resection in 62 patients. They encountered bile leakage in two patients, and secondary hemorrhage in one patient in the collagen group, while no such complications were observed in fibrin sealant administered group[26]. Even though the study of Figueras *et al*[27] could not justify the influence of fibrin sealant, many clinical studies are available in the literature showing the useful effects of fibrin sealant in prevention of bile leakage after hepatic resection[27-31].

In conclusion, fibrin sealant may be preferred over to primary suture in appropriate cases of liver trauma, due to the shorter bleeding time, faster regeneration and lesser intra-abdominal adhesions by its use. However, more studies focusing on the clarification of the effects of this product on liver regeneration are needed to check the validity of these results.

COMMENTS

Background

Liver injuries occur as a result of blunt and penetrating traumas and rarely due to some iatrogenic reasons. In blunt abdominal traumas, liver turns out to be the organ that mostly gets injured. Acute bleedings and operative complications cause an increase in rate of mortality and morbidity.

Research frontiers

Fibrin sealant can be used as a safe and appropriate treatment technique in

liver injuries. There were many clinical and experimental studies in the literature showing the useful effects of fibrin sealant in prevention of bile leakage and in shortening bleeding time after hepatic resection and liver injury. However, the effects on liver regeneration and development of intra-abdominal adhesions yet remain unclear.

Innovations and breakthroughs

In this experimental study, more intra-abdominal adhesions were observed in the primary suture group compared to the fibrin sealant group. Hepatic healing was faster and liver abscess was not observed in the fibrin sealant group postoperatively. Minimal tissue damage and non-existent ischemia in liver parenchyma ensured with application of this technique.

Applications

Fibrin sealant can be used clinically in appropriate cases including liver trauma or hepatic resection.

Terminology

The fibrin sealant kit contains proteins and thrombin, properly cooled and dried, as well as a calcium chloride and aprotinin solution. Mixing these substances and covering the tissue surface result a thrombin solution.

Peer review

In this experimental study, the authors revealed that fibrin sealant causes less intra-abdominal adhesions and faster hepatic healing while allowing shorter hemostasis time compared to the primary suture as assessed in experimental liver trauma. Therefore, fibrin sealant can be preferred over primary suture in appropriate cases, including liver trauma.

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RAPID COMMUNICATION

C-reactive protein levels during a relapse of Crohn's disease are associated with the clinical course of the disease

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Abstract

AIM: To explore if C-reactive protein (CRP) levels might serve as a prognostic factor with respect to the clinical course of Crohn's disease and might be useful for classification.

METHODS: In this retrospective cohort study we enrolled 94 patients from the inflammatory bowel disease (IBD) database of the University Medical Centre Utrecht. CRP levels during relapse were correlated with the number of relapses per year. Severity of relapses was based on endoscopic reports and prednisone use. Furthermore, patients were categorized in a low or high CRP group based on their CRP response during relapse and demographic and clinical features were compared.

RESULTS: Overall, a positive correlation between CRP levels, number of relapses, and severity of relapse was found (respectively r_s = 0.31, P < 0.01 and r_s = 0.50, P < 0.001). Employing a cut-off level of 15 mg/L, the index CRP level was found to discriminate patients with respect to the number of relapses per year, as well as for severity of relapses (respectively 0.25 ± 0.16 *vs* 0.36 ± 0.24, P < 0.05 and 4.4 ± 1.2 *vs* 3.2 ± 1.1 on a 10-point visual analogue scale, P < 0.001 for the high CRP and low CRP groups respectively). In addition, the high CRP group showed more cumulative days of prednisone use per year (107 ± 95 *vs* 58 ± 48, P < 0.05), as well as a better response to infliximab (93 % *vs* 33 %, P = 0.06).

CONCLUSION: A higher CRP level during relapse seems to be associated with a more severe clinical course of disease.

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Key words: Crohn's disease; Infliximab; C-reactive protein; Prognosis; Clinical behaviour

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INTRODUCTION

Crohn's disease (CD) is thought to result from an ongoing activation of the mucosal immune system leading to an inappropriate innate immune response to normal luminal factors in a genetically susceptible individual[1,2]. The clinical expression of CD is heterogeneous with a wide spectrum of patterns and different clinical courses. Classification of CD is, therefore, difficult[3]. In the Vienna Classification, distinct definitions to categorize patients with CD have been formulated[4]. This classification distinguishes between age (< or > 40 years), anatomical localization (terminal ileum, colon, ileocolon, upper gastrointestinal) and disease behaviour (non-stricturing/non-penetrating, stricturing and penetrating). However, it has been shown to be unreliable in predicting the course of the disease[5,6]. Currently, it is not known whether other tools, such as the recently developed Montreal classification will prove to be superior in this respect[7,8].

C-reactive protein (CRP), an acute phaseprotein produced by hepatocytes upon activation by proinflammatory cytokines such as IL-6, IL-1α and tumour necrosis factor-alpha (TNF-α) is widely used as a parameter of inflammatory activity in a variety of infectious and inflammatory diseases[9-11]. Moreover, it has been found to correlate with clinical parameters of disease activity in CD[12-18].

We hypothesized that a high serum CRP level during a relapse predisposes patients to a more severe course of the disease. In the present study, we studied retrospectively the putative association between the height of CRP levels during a relapse and the clinical course of disease in patients with CD.

MATERIALS AND METHODS

In this retrospective cohort study, 94 patients with CD were selected from the IBD database of the University Medical Centre Utrecht, comprising the records of all patients diagnosed with IBD in the period 1994-2005.

CD patients were eligible if they were 18 years of age or older, had experienced at least one well-documented relapse during which serum CRP values were determined, and from whom records over a period of at least 4 years of follow-up were available. Excluded were patients with other chronic inflammatory diseases, concomitant infections or other serious comorbidity.

Data were collected from electronic and clinical charts. The index CRP level used for analysis had to be measured during a relapse which was confirmed by (ileo) colonoscopy and/or a small bowel follow-through examination. The highest CRP level during this exacerbation, within the first 4 wk after the onset of symptoms, was taken as an index CRP level. Severity of the index exacerbation was assessed retrospectively by two independent, blinded reviewers based on the endoscopic reports and recorded on a 10-point visual analogue scale (VAS). The mean of both scores was used for analysis.

The total number of relapses during follow up was assessed. These relapses were defined as a period of inflammatory activity accompanied by progressive symptomatology which was confirmed by (ileo) colonoscopy and/or a small bowel follow-through examination and requiring hospitalization and/or initiation of treatment with steroids or other immunosuppressive drugs. Another primary endpoint was the use of prednisone, measured in number of days during follow up.

Furthermore, age at the time of the diagnosis of CD, duration of the disease, localization and behaviour of CD according to the Vienna Classification, the presence of granulomas in pathology specimens, smoking behaviour, surgical treatment and treatment with immunosuppressives other than prednisone (azathioprine, mesalazine, methotrexate, budesonide, cortisone enema's or infliximab) were noted.

Statistical analysis

Analysis was performed using SPSS for Windows, version 12.0. The correlation between the index CRP value and the primary outcome measures were calculated employing a bivariate correlation with a non-parametric distribution (Spearman's rho). Differences in the primary, as well as the secondary outcomes of the Low C-reactive protein subgroup (LCRP) and High C-reactive protein subgroup (HCRP) groups were calculated with the Mann Whitney *U* test to compare means (non-parametric distribution) and the chi-square test to compare the differences in distribution (non-parametric distribution).

All tests were 2-tailed analysed. Data are presented in mean ± SD or otherwise stated. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Correlation variables

For the whole group, a significant positive correlation between the index CRP level and the number of relapses per year was noted ($r_s = 0.31$, $P < 0.01$, Figure 1). Severity of relapses was correlated with the index CRP level as well ($r_s = 0.50$, $P < 0.001$). The severity was based on the mean score of both independent reviewers with an interobserver correlation of $r_s = 0.71$, $P < 0.001$. The correlation between the index CRP level and cumulative use of prednisone per year was small but significant, $r_s = 0.22$, $P < 0.05$.

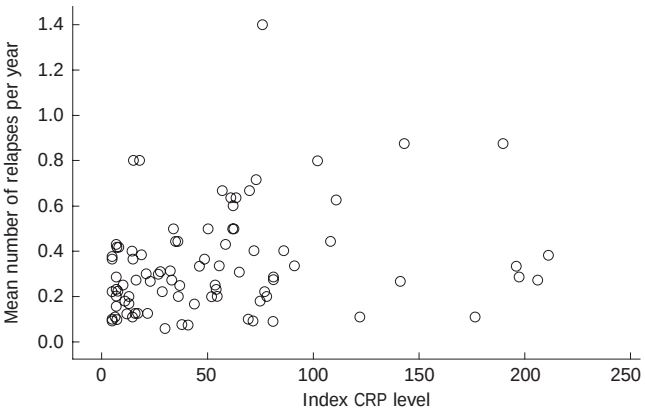


Figure 1 Correlation between the index CRP and the mean number of relapses per year. Spermann's rho = 0.31, $P < 0.01$.

Comparison of subgroups

Based on the index CRP levels two groups were created, the LCRP and HCRP group, with a cut-off level of 15 mg/L. Twenty-seven patients had a serum CRP level of ≤ 15 mg/L and 67 had a CRP level of > 15 mg/L. Baseline characteristics of the subgroups are given in Table 1. The baseline characteristics of the groups were similar. There were no differences between the two groups regarding gender distribution, mean age, age at the time of diagnosis, disease localization and disease behaviour according to the Vienna classification.

Table 2 shows the clinical findings, drug use, surgeries and related factors in the two groups. The numbers of relapses were respectively 0.25 ± 0.16 and 0.36 ± 0.24 per year in the LCRP and HCRP group ($P < 0.05$). The mean severity of the index exacerbation on a 10-point VAS was 4.4 ± 1.2 in the HCRP and 3.2 ± 1.1 in the LCRP group ($P < 0.001$). The number of courses of prednisone did not differ between the groups, but patients in the HCRP group were treated significantly longer with prednisone than LCRP-patients, 107 ± 95 *vs* 58 ± 48 d per year ($P < 0.05$).

There was no significant difference in the number of patients on infliximab in both groups, 20.9 % in the HCRP and 11.1% in the LCRP group, but the response rate was higher in the HCRP subgroup compared to the LCRP subgroup, respectively 92.8% *vs* 33.3%, $P = 0.06$.

In the follow-up of patients in the HCRP and LCRP groups, the percentages of azathioprine (77.6 *vs* 66.7), mesalazine (98.5 *vs* 100), budesonide (71.6 *vs* 63.0), cortisone enema's (43.3 *vs* 44.4) and methotrexate (4.5 *vs* 3.7) use did not differ significantly, nor did the total number of CD-related surgeries [1.7 (SD 0.1) *vs* 2.0 (SD 0.6) per patient], construction of stomas (22.4 % *vs* 22.2 %) or development of fistulas and/or abscesses (26.9 % *vs* 18.5%).

There were no significant differences between smokers and non-smokers. Patients with granulomas were significantly younger (38.4 ± 8.5 *vs* 45.4 ± 13.3 years, $P < 0.05$) and tended to be younger at the time

Table 1 Baseline characteristics of patients with an index serum C-reactive protein (CRP) level ≤ 15 and > 15 mg/L

Index serum CRP level during relapse (mg/L)	≤ 15	> 15	*P*-value
	LCRP	HCRP	
n	27	67	
Time of follow-up, yr (SD)	10.8 (4.0)	10.3 (3.7)	NS
Demographic factors			
Sex: men	12 (44.4%)	32 (47.7%)	NS
Mean age, yr (SD)	45 (13.5)	42 (11.0)	NS
Clinical features			
Age at time of diagnosis (SD)	27.0 (8.3)	27.4 (10.8)	NS
Duration of disease, yr (SD)	18.0 (10.2)	14.3 (7.3)	NS
Localization			
Terminal ileum	6 (22.2%)	8 (11.9%)	NS
Colon	7 (25.9%)	31 (46.2%)	
Ileocolon	13 (48.1%)	26 (38.8%)	
Upper gastrointestinal	1 (3.7%)	2 (3.0%)	
Behaviour			
Non-stricturing non penetrating	13 (48.1%)	26 (38.8%)	NS
Stricturing	7 (25.9%)	15 (22.4%)	
Penetrating	7 (25.9%)	26 (38.8%)	

Data are given in numbers and percentages, unless shown otherwise. Significance between the two groups is calculated. Localization and behaviour according to the Vienna Classification of Crohn's disease. HCRP: High CRP response; LCRP: Low CRP response; NS: No significance; SD: Standard deviation.

Table 2 Subgroups with an index serum C-reactive protein (CRP) level of ≤ 15 and > 15 mg/L, comparison of clinical findings, drug use, surgeries and related factors

Index serum CRP level during relapse (mg/L)	≤ 15	> 15	*P*-value
	LCRP	HCRP	
Clinical findings			
Mean number of relapses per year (SD)	0.25 (0.16)	0.36 (0.24)	< 0.05
Mean severity of the index relapse according to endoscopic reports, on a 10 points visual analogue scale (SD)	3.18 (1.09)	4.39 (1.24)	< 0.001
Drug use			
Number of patients with prednisone use	23 (85.2%)	62 (92.5%)	NS
Mean number of days of prednisone use per year (SD)[1]	58 (48)	107 (95)	< 0.05
Surgeries			
Number of patients who underwent ≥ 1 surgeries	12 (44.4%)	27 (37.3)	NS
Total number of surgeries[2,3]	2.0 (0.6)	1.7 (0.09)	NS
Related factors			
Smoking: *n*	23	58	
smokers	8 (34.8%)	24 (41.4%)	NS
non-smokers	11 (47.8%)	24 (41.4%)	
quitted smokers	4 (17.4%)	10 (17.2%)	
Presence of granulomas: *n*	22	51	
	8 (36.4%)	25 (49.0%)	NS

[1]Number of days are based on the mean number of months per year; [2]Calculated mean is based on these patients who underwent surgery; [3]Total of ileocecalresection, partial ileum resection, partial colon resection, (sub) total colectomy and proctectomy. HCRP: High CRP response; LCRP: Low CRP response; NS: No significance; SD: Standard deviation. Data are given in number and percentage, unless shown otherwise.

of diagnosis (24.2 ± 5.9 *vs* 29.7 ± 12.1 years, $P = 0.09$) compared with patients without granulomas. Other differences were not detected.

DISCUSSION

We hypothesized that the CRP response during a relapse of CD discriminates between phenotypic subsets of patients with an aggressive or more benign course of disease and predicts the response to drugs. We found a significant but relatively modest correlation between level of CRP during the index exacerbation and the number, as well as the severity of relapses per year. A significant, but rather small, correlation between CRP level and prednisone use was revealed. LCRP and HCRP groups were found to be comparable regarding baseline characteristics but the number and severity of relapses was significantly higher in HCRP patients. Furthermore, patients in the HCRP group appeared to respond better to infliximab infusions. The latter phenomenon is in line with literature. This apparently more aggressive disease behaviour in the HCRP patients was not reflected in a higher frequency of drug use other than prednisone. A possible explanation is the tertiary referral setting of this study, resulting in enrolment of relative severe cases of CD in most of whom immunomodulators were already prescribed.

Interestingly, we found the index CRP and severity of relapse based on endoscopic reports to be correlated. CRP levels are found to be weakly associated with clinical activity indices by several authors[14,19-21], although this has not been reported consistently[22]. Colombel *et al* reported a striking correlation of CRP levels with radiologic findings of perienteric inflammation[12]. Whether involvement of mesenteric adipocytes in the immune response results in an increased CRP level and, in the long run, the course of disease, is a matter of speculation. We could not confirm the data from Floren *et al*, who reported an exclusive ileal disease distribution in a group of CD patients with a CRP level below the level of 10 mg/L. In this study the LCRP group had a significant lower body mass index as well, while no differences were found in the frequency and distribution of CARD15 variants[23]. The association of colonic disease phenotype, cigarette smoking or the presence of granulomas with relapses as reported in other studies was not reproduced in the current study either[24-30].

Our study has its limitations. The severity of index relapses was scored retrospectively and the presence of relapses during the follow-up period was assumed if certain criteria were met in the charts. We tried to overcome these limitations by using blinded assessment of the index colonoscopies by two gastroenterologists and by defining strict criteria for relapse in the follow-up phase. The number of courses of prednisone had to be scored retrospectively as well, which only gives a global impression. In addition, we had to choose a cut-off level of CRP to create two groups. In literature, there is only one comparable study[23] in which 10 mg/L was used as the cut-off level. We found that a CRP level of 15 mg/L discriminated the two groups optimally.

In conclusion, this study showed that the level of CRP

during a relapse of CD can serve as a prognostic factor for the number and severity of relapses. Patients with a CRP level > 15 mg/L experienced more and more severe relapses, were treated more extensively with prednisone and responded better to infliximab therapy. However, we consider these differences too small to be of use in clinical decision making.

COMMENTS

Background

Crohn's disease (CD) may affect any part of the gastrointestinal tract and is characterized by a broad spectrum of clinical presentations and a variable disease course. Disease flares occur randomly and cannot be predicted reliably. The aim of the present study was to explore the value of C-reactive protein (CRP) as a prognostic factor with respect to the clinical course of CD.

Research frontiers

Early identification of CD patients with a propensity to an aggressive or more benign disease course could be of major clinical importance; this might guide the clinician in deciding what maintenance therapy should be prescribed and could even result in early initiation of aggressive induction therapy to avoid flares or complications. To date, a simple parameter, reliably predicting disease activity and flares is not available. From all laboratory markers, CRP seems to be the most promising candidate in this respect. Not all patients with confirmed disease activity, however, display raised CRP levels. We hypothesized that this group constitutes a distinct, benign phenotype, possibly requiring less intensive treatment.

Innovations and breakthroughs

A high CRP level during relapse is positively correlated with the severity and frequency of relapses. Furthermore, this was found to be associated with longer courses of steroids and a better response following infliximab administration.

Applications

These findings suggest that phenotypes can be identified using a biochemical parameter such as CRP, which predicts to a certain extent the course of disease and response to drugs. The differences found in this study, however, are too small to guide clinical decision making and the use of CRP in this setting should be seen as an additive tool.

Terminology

High CRP and low CRP responders are patients that respectively react with a high CRP level and a low CRP level during a relapse of Crohn's Disease.

Peer review

This is an important contribution to the literature of inflammatory bowel disease. The study is well conceived and the report is well written.

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RAPID COMMUNICATION

Are there tumor suppressor genes on chromosome 4p in sporadic colorectal carcinoma?

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Abstract

AIM: To study the candidate tumor suppressor genes (TSG) on chromosome 4p by detecting the high frequency of loss of heterozygosity (LOH) in sporadic colorectal carcinoma in Chinese patients.

METHODS: Seven fluorescent labeled polymorphic microsatellite markers were analyzed in 83 cases of colorectal carcinoma and matched normal tissue DNA by PCR. PCR products were eletrophoresed on an ABI 377 DNA sequencer. Genescan 3.7 and Genotype 3.7 software were used for LOH scanning and analysis. The same procedure was performed by the other six microsatellite markers spanning D4S3013 locus to make further detailed deletion mapping. Comparison between LOH frequency and clinicopathological factors was performed by χ^2 test.

RESULTS: Data were collected from all informative loci. The average LOH frequency on 4p was 24.25%, and 42.3% and 35.62% on D4S405 and D4S3013 locus, respectively. Adjacent markers of D4S3013 displayed a low LOH frequency (< 30%) by detailed deletion mapping. Significant opposite difference was observed between LOH frequency and tumor diameter on D4S412 and D4S1546 locus (0% *vs* 16.67%, P = 0.041; 54.55% *vs* 11.11%, P = 0.034, respectively). On D4S403 locus, LOH was significantly associated with tumor gross pattern (11.11%, 0, 33.33%, P = 0.030). No relationship was detected on other loci compared with clinicopathological features.

CONCLUSION: By deletion mapping, two obvious high frequency LOH regions spanning D4S3013 (4p15.2) and D4S405 (4p14) locus are detected. Candidate TSG, which is involved in carcinogenesis and progression of sporadic colorectal carcinoma on chromosome 4p, may be located between D4S3017 and D4S2933 (about 1.7 cm).

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Key words: Loss of heterozygosity; Colorectal carcinoma; Chromosome 4p; Tumor suppressor gene

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INTRODUCTION

Colorectal cancer (CRC) constitutes the second most common neoplasm in Western countries and is the third leading cause of cancer-related death, the overall 5-year survival rate is approximately 45%[1]. Improvement in its prognosis can not be achieved without a better understanding of its etiology and tumor molecular biology. In recent years, the genetic basis of human tumors has been increasingly elucidated. As a model for both multistep and multipathway carcinogenesis, colorectal neoplastic progression provide paradigms of both oncogenes and tumor suppressor gene in epithelial tumors[2,3]. The latter changes predominate. In addition to the allelic loss on chromosome 5q, 17p and 18q, many other chromosome losses can be observed in colorectal carcinoma. Regions on chromosome 1q, 4p, 6p, 6q, 8p, 9p and 22q were lost in 25%-50% of the colorectal tumor cases studied previously[2].

Chromosome losses in colorectal tumor were first detected by cytogenesis, later, by probes of restriction fragment length polymorphisms (RFLP) and now by loss of heterozygosity (LOH) in analyzing allelic loss. The loss of tumor suppressor genes is believed to be one of the key steps to carcinogenesis of colorectal cancer[4]. The loss of one allelic at specific locus is caused by deletion mutation or loss of a chromosome from a chromosome pair[5]. When this occurs at a tumor suppressor gene locus where one of the allelics is already abnormal, it can result

in neoplastic transformation. The LOH analysis based on polymorphic microsatellite DNA has become an effective and powerful tool currently to find informative loci and candidate tumor suppressor genes[6,7]. Most investigations concentrated on defining the minimal regions of loss of specific chromosomes in various cancers in an effort to identify the putative tumor suppressor genes targeted by the loss[8].

In this study, we first analyzed the LOH events on chromosome 4p using seven microsatellite markers and made further refined deletion mapping analysis spanning D4S3013 locus in 83 sporadic colorectal carcinoma cases in an attempt to identify additional candidate tumor suppressor genes involved in colorectal tumorigenesis.

MATERIALS AND METHODS

Patient sample and DNA extraction

This study was based on consecutively collected tumors in 83 patients with colorectal cancer, including 40 males and 43 females, treated at the surgical department in Shanghai First People's Hospital, China. The patients' ages ranged from 31 to 84 years with a median of 66. The cancerous tissue and adjacent normal control tissue (> 10 cm) were freshly frozen. The tissues were cut into cubes of approximately 2 mm^3 and immediately frozen in liquid nitrogen. Each patient gave his or her informed consent for the use of his or her tissue in this study. DNA was extracted using standard methods with proteinase K digestion and phenol/chloroform purification[9]. All patients were confirmed by pathology and were staged by Duke's criteria.

Microsatellite markers and PCR

Initially, 83 cases of colorectal cancer were analyzed by PCR using seven microsatellite markers (Shanghai Biology Technology Company, China) which map to chromosome 4p. DNA samples were analyzed as matched normal and tumor pairs using primers of the following microsatellite loci (hereditary location/heterozygote): pter-D4S412 (4p16.3/76)-D4S2935 (4p16.1/62)-D4S1599 (4p16.1/81)-D4S303 (4p15.33/76)-D4S3013 (4p15.2/84)-D4S391 (4p15.2/85)-D4S405 (4p14/85). The average hereditary distance was 8.65 cm[10] (Figure 1A). As the D4S3013 locus showed high LOH frequency (35.62%), six additional microsatellite markers map to chromosome 4p15 were employed to further investigate LOH. The same DNA samples were then analyzed as matched pairs for the following microsatellite markers (location/heterozygote): pter-D4S2926 (4p15.32/80)-D4S1546 (4p 15.31 /77) -D4S3017 (4p 15.31/82)-D4S2933 (4p 15.31/60)-D4S2948 (4p 15.2 /81)-D4S1551 (4p 15.2/78). The average hereditary distance was restricted within 1.03 cm[10] (Figure 1B).

LOH result analysis

A portion of each PCR product (0.5 μL) was combined with 0.1 μL Genescan 500 size standard (PE Applied Biosystems Foster City, CA, USA) and 0.9 μL formamide loading buffer. After denaturation at 96℃ for 5 min, products were eletrophoresed on 5% polyacrylamide

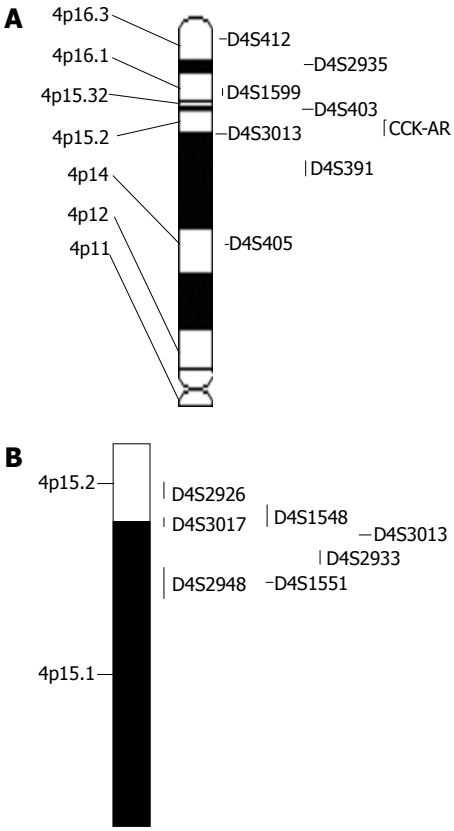


Figure 1 **A:** Microsatellite markers and candidate tumor suppressor genes on 4p (www.gdb.org); **B:** Microsatellite markers on D4S3013 (www.gdb.org).

gels using an ABI 377 DNA sequencer (PE Applied Biosystems Foster City, CA, USA) for 2.5 h. Genotype 3.7 software display individual gel lanes as electropherograms with a given size, height, and area for each detected fluorescent peak. Stringent criteria were used to score the samples. Alleles were defined as the two highest peaks within the expected size range. A ratio of T1:T2/N1:N2 of less than 0.67 or greater than 1.50 was scored as a loss of heterozygosity (Figure 2). Most amplified normal DNA produced two PCR products indicating heterozygosity. A single fragment amplified from normal DNA (homozygosity) and those PCR reactions, in which fragments were not clearly amplified, were scored as not informative. The LOH frequency of a locus is equal to the ratio of the number between allelic loss and informative cases. The average LOH frequency of chromosome 4p is the average value of each locus.

Statistical analysis

Comparisons between LOH and clinicopathological data were performed by χ^2 test. $P < 0.05$ was considered as statistically significant.

RESULTS

LOH analysis of colorectal cancer on 4p

Eighty-three colorectal cancers were analyzed for LOH at the seven marker loci spanning chromosome 4p. All loci got informative messengers. The average LOH frequency on 4p was 24.25%. Sixty-three samples (75.90%) showed

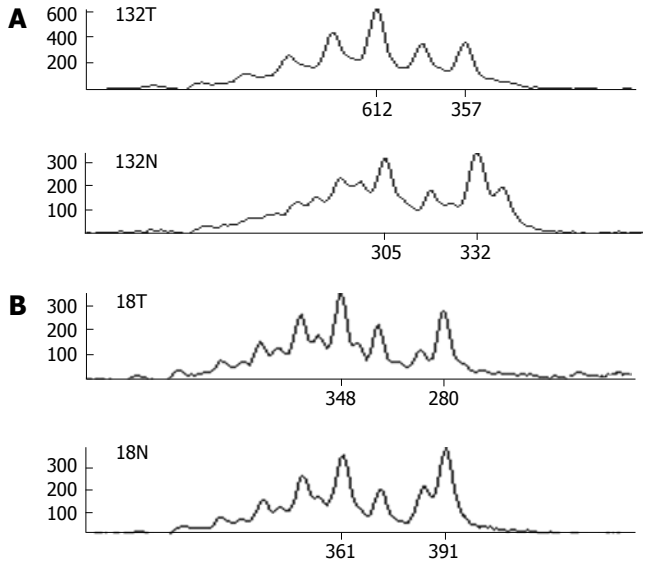


Figure 2 LOH Demonstration. **A:** Classic LOH peak: Allele ratio = (T1/T2)/(N1/N2) = (612/357)/(305/332) = 1.87 > 1.5; **B:** Heterozygosity retention: Allele ratio = (T1/T2)/(N1/N2) = (348/280)/(361/391) = 0.67 < 1.34 < 1.5. T: Tumor; N: Normal.

Table 1 LOH result and hereditary distance/location of chromosome 4p

Locus	Location	LOH cases	Normal cases	Informative rate	Distance (cM)	LOH rate (%)
D4S412	4p16.3	5	57	74.7	-	8.06
D4S2935	4p16.1	24	55	95.18	7.4	30.38
D4S1599	4p16.1	9	28	44.58	10.8	24.32
D4S403	4p15.33	3	34	44.58	5.1	8.1
D4S3013	4p15.2	26	47	86.9	7.7	35.62
D4S391	4p15.2	13	46	71.84	7.4	22.03
D4S405	4p14	22	30	62.65	13.4	42.3

at least one LOH event. Two distinct regions of frequent allelic loss at D4S3013 (4p15.2) and D4S405 (4p14) locus on chromosome were detected (Table 1). The LOH frequency was 35.62% and 42.3%, respectively. This suggested that putative tumor suppressor genes may be located near D4S3013 and D4S405 loci.

LOH deletion mapping results on 4p15 encompassing D4S3013

The chromosome region spanning D4S3013 locus on 4p15 was investigated using a saturation mapping strategy with another 6 microsatellite markers that are closely located within this region (Figure 2). To detect putative tumor suppressor genes easily, we limited average hereditary distance to 1.03 cm. Forty samples (48.19%) showed at least one LOH event. The average LOH frequency spanning D4S3013 was 24.2% (Table 2). We found that adjacent markers of D4S3013 displayed a low LOH frequency (< 30%), especially on D4S2933 locus, much less information was obtained because of more homozygosity.

Relationship between clinicopathological features and LOH on 4p

On D4S412 locus, none LOH was detected in patients with

Table 2 LOH result and hereditary distance/location of detailed deletion mapping spanning D4S3013

Locus	Location	LOH cases	Normal cases	Informative rate	Distance (cM)	LOH rate (%)
D4S2926	4p15.32	7	38	54.22	-	15.56
D4S1546	4p15.31	9	38	56.63	1.6	19.15
D4S3017	4p15.31	13	46	71.08	0.5	22.03
D4S3013	4p15.2	26	47	87.95	1.2	35.62
D4S2933	4p15.31	2	14	19.28	0.5	12.5
D4S2948	4p15.2	15	49	77.11	1.9	23.44
D4S1551	4p15.2	12	64	91.57	0.5	21.05

tumor larger than 5 cm in diameter (0/27), while in patients with tumor less than 5 cm in diameter, LOH frequency was 14.29% (5/35, $P = 0.041$). On the contrary, on D4S1546 locus, LOH frequency was 35.29% (6/17) in the former; and only 10% (3/30) in the latter locus ($P = 0.030$). Notably, on D4S403 locus, LOH was significantly associated with tumor gross pattern. In tumor of the massive, ulcerative and encroaching pattern, the LOH frequency was 10%, 0%, 33.33%, respectively ($P = 0.030$). No significant relationship was found between clinicopathological features and LOH on other loci (data not shown).

DISCUSSION

Inactivation of tumor suppressor genes appears to be one of the genetic mechanisms involved in the development of colorectal cancer[11,12]. Deletion of tumor suppressor genes occur frequently in human malignances. Such events can be detected using markers from the region of genome that include a tumor suppressor gene. Allelic deletions detected as LOH have been proved useful for mapping regions of DNA that contains tumor suppressor genes, i.e., LOH at specific chromosomal regions strongly suggests the existence of tumor suppressor genes at the relevant segment.

A great deal of evidence supported the presence of tumor suppressor genes in the short arm of chromosome 4. These include the reversion of the immortal phenotype by chromosome 4 transfer[13] and the frequent occurrence of losses in or near the 4p14-4p16 region in bladder cancer[14]. LOH has been observed at distal 4p in sporadic neuroblastoma with an incidence raging from 20% to 29%[15,16]. Using array comparative genomic hybridization, Hurst *et al*[17] reported the loss frequency of 4p to be 52% in bladder cancer. More importantly, Shirapurkar *et al*[18] observed the loss frequency of > 50% at 4p15.1-4p15.3 in malignant mesothelioma and lung carcinoma. LOH on 4p was 21% and > 30% in differentiated adenocarcinoma of stomach as well[19,20]. Head and neck squamous cell carcinoma, invasive cervical cancer and acinic cell carcinoma also showed a high allelic loss frequency[21-23].

In colorectal tumors, previous alletic typing[24], cytogenetic[25-27] and comparative genomic hybridization[28] studies have reported moderate losses(0%-30%) of chromosome 4. These data have not raised special interest in this chromosome as a candidate to harbor a tumor suppressor gene, therefore, colorectal cancer investigations

have not included a detailed analysis of loss in this chromosome. Choi *et al*[29] reported a LOH frequency of 24%-30% at just several loci on chromosome 4 in colorectal cancer. Later, Arribas *et al*[30,31], used AP-PCR method and suggested chromosome 4p14-4p16 may contain tumor suppressor gene, because LOH frequency on D4S2397 was as high as 35%. These reports indicate that 4p14-4p16 region displayed frequent loss in a couple of cancers, so 4p14-4p16 region is of important value for TSG screening.

D4S3013 locus region, 4p15.2, was concordant with several reports in other tumors before[14,16,20,21]. In this study, we investigated the LOH on 4p in 83 sporadic cases of colorectal cancer. The results showed putative tumor suppressor gene may harbor adjacent to D4S405 and D4S3013 locus. We made further detailed deletion mapping spanning D4S3013 locus, and found that the surrounding markers of D4S3013 displayed a low LOH frequency (< 30%). Therefore, we speculate that the candidate TSG may be located between D4S3017 and D4S2933, about 1.7 cm in hereditary distance.

We found several loci were significantly associated with clinicopathological features. On D4S412 locus, no LOH was detected in patients with tumor larger than 5 cm in diameter, while in patients with tumor less than 5 cm in diameter, the LOH frequency was 14.29% ($P = 0.041$). On the contrary, on D4S1546 locus, the LOH frequency showed opposite phenomenon. On D4S403 locus, LOH was significantly associated with tumor gross pattern. Similarly, Arribas *et al*[31] found solely at the D4S2397 locus was indicative of a shorter disease-free survival ($P = 0.027$). Choi *et al*[29] found 4p loss was significantly associated with early onset of colorectal cancer. The effect of 4p loss on the early-onset disease is unlikely to be the result of tumor aggressiveness, because 4p loss was not found to be correlated with cancer-related death. Nishizuka *et al*[20] found 4p LOH had an essentially similar frequency in early and advanced differentiated adenocarcinoma. The differential behavior of LOH at different markers suggested that distinct mechanisms and/or selection presses participate in the mutational event that affect this chromosomal region during the tumorigenic process.

Regarding allelic loss at 4p, cholecystokinin type A receptor (CCK-AR) gene maps near D4S2397[32,33] (Figure 1). Recent reports have suggested that cholecystokinin receptor may function as a tumor suppressor gene[34,35].

In summary, we investigated LOH on 4p in sporadic colorectal carcinoma in Chinese patients and detected two high deletion regions encompassing D4S3013 (4p15.2) and D4S405 (4p14). Candidate TSG, involved in sporadic colorectal carcinoma on chromosome 4p, may be located between D4S3017 and D4S2933 (about 1.7 cm). Further related gene screening and functional studies may contribute to the identification of the tumor suppressor gene in these regions.

COMMENTS

Background

Cancer arises from the accumulation of inherited polymorphism (i.e. SNPs) and mutation and/or sporadic somatic polymorphism (i.e. non-germline polymorphism) in cell cycle, DNA repair, and growth signaling genes. Neoplastic progression is generally characterized by the accumulation of multiple somatic-cell genetic alterations as the tumor progresses to advanced stages. The classic mechanism of tumor suppressor gene inactivation is described by two-hit modes in which one allele is mutated (or promoter hypermethylation or a small intragenic deletion) and the other allele is lost through a number of possible mechanisms, resulting in loss of heterozygosity at multiple loci. Loss of heterozygosity is the most common molecular genetic alteration observed in human cancers. In the model of colorectal tumorigenesis, mutational inactivation of tumor suppressor genes predominates.

Research frontiers

Most genome-wide scans for loss of heterozygosity (LOH) have been conducted at low resolution with a relatively small number of polymorphic markers. For example, an average of 120 microsatellites have been used to determine the allelotype of multiple different human neoplasms in a series of studies since 1995, and the highest density microsatellite allelotype was about 280 polymorphic markers before the year 2000. SNPs are the most common form of sequence variation in human genome, occurring approximately every 1200 base pairs (bps). High density mapping of genetic losses reveals potential tumor suppressor loci and might be useful for clinical classification of individual tumors. SNP array has been introduced recently for genome-wide screening of chromosome imbalance. Higher density SNP array can effectively detect small regions of chromosomal changes and provide more information regarding the boundaries of loss regions.

Innovations and breakthroughs

A great deal of evidence supported the presence of tumor suppressor genes in the short arm of chromosome 4. Much less studies have been reported in colorectal cancer. Previous allelotyping analysis of cancer by many groups was used with a relatively low density of markers. By deletion dense markers mapping, we detected two obvious high frequency LOH regions spanning D4S3013 and D4S405 locus in colorectal cancer. Candidate TSG, might be located between D4S3017 and D4S2933 (about 1.7 cm).

Applications

We used this method to detect some major allelic loss regions in genome-wide scans of LOH in patients with colorectal cancer.

Terminology

LOH is caused by a variety of genetic mechanisms, including physical deletion of chromosome non-disjunction and mitotic non-disjunction followed by republication of the remaining chromosomes, mitotic recombination and gene conversion. The mechanisms of LOH are remarkably chromosome-specific. Some chromosomes display a complete loss. However, more than half of the losses are associated with a only partial loss of a chromosome rather than a whole chromosome. LOH is also a common form of allelic imbalance and the detection of LOH has been used to identify genomic regions that harbor tumor suppressor genes and to characterize different tumor types, pathological stages and progression.

Peer review

This is a report that describes the LOH events in sporadic colorectal cancer in Chinese patients, further studies must benefit from this paper. The data presented is clear and concise in the text.

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RAPID COMMUNICATION

A paradox: Insulin inhibits expression and secretion of resistin which induces insulin resistance

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Abstract

AIM: To confirm whether insulin regulates resistin expression and secretion during differentiation of 3T3-L1 preadipocytes and the relationship of resistin with insulin resistance both *in vivo* and *in vitro*.

METHODS: Supernatant resistin was measured during differentiation of 3T3-L1 preadipocytes. L6 rat myoblasts and hepatoma cell line H4IIE were used to confirm the cellular function of resistin. Diet-induced obese rats were used as an insulin resistance model to study the relationship of resistin with insulin resistance.

RESULTS: Resistin expression and secretion were enhanced during differentiation 3T3-L1 preadipocytes. This cellular differentiation stimulated resistin expression and secretion, but was suppressed by insulin. Resistin also induced insulin resistance in H4IIE hepatocytes and L6 myoblasts. In diet-induced obese rats, serum resistin levels were negatively correlated with insulin sensitivity, but not with serum insulin.

CONCLUSION: Insulin can inhibit resistin expression and secretion *in vitro*, but insulin is not a major regulator of resistin *in vivo*. Fat tissue mass affects insulin sensitivity by altering the expression and secretion of resistin.

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Key words: Resistin; Insulin; Insulin resistance

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INTRODUCTION

Obesity is a worldwide health problem directly linked to several disease processes such as hypertension and type 2 diabetes mellitus[1]. Adipose tissue is not only an organ for passive energy reserve, but also an active endocrine organ secreting a wide range of hormones and other protein factors called adipokines[1,2]. Among the adipokines, resistin is involved in insulin sensitivity and glucose tolerance[3,4] while others are involved in hemostasis, inflammatory and stress responses, and energy balance[5,6].

Resistin, initially identified in screening for adipocyte-specific transcripts down-regulated by treatment with thiozolidinedione (TZDs), belongs to a novel family of cysteine-rich proteins, each with a unique tissue distribution[3, 4]. In rodents, resistin predominantly expressed in white adipose tissue[4] reduces insulin sensitivity in adipocytes and skeletal muscles by impairing insulin-mediated glucose transport and inducing the expression of suppressor of cytokine signaling 3 (SOCS3)[7-9], and regulates fasting blood glucose by increasing hepatic glucose release[10]. Therefore, resistin might provide a link between obesity and diabetes mellitus.

Initial studies on the regulation of resistin indicate that resistin expression is reduced by fasting and increases rapidly on refeeding[3]. Circulating resistin levels are elevated in genetically obese (ob/ob, db/db) mice, and obese is induced by a high-fat diet[3]. Insulin inhibits resistin mRNA expression in 3T3-L1 preadipocytes[11,12]. However, these data do not support a role of resistin in insulin resistance[11]. If resistin is mainly regulated by insulin, the major function of resistin is to induce insulin resistance, forming an insulin-resistin-insulin sensitivity positive feedback loop that cannot exist *in vivo*.

In the present study, resistin expression and secretion were elevated during 3T3-differentiation of L1

preadipocytes. This cellular differentiation-stimulated resistin expression and secretion were suppressed by insulin. Resistin also induced insulin resistance in H4IIE hepatocytes and L6 myoblasts. In diet-induced obese rats, serum resistin levels were negatively correlated with the insulin sensitivity index (ISI). No negative correlation was found between the levels of fasting serum insulin and resistin, suggesting that insulin is not the major regulator of resistin in rodents.

MATERIALS AND METHODS

Cell culture

3T3-L1 preadipocytes were cultured at 37℃ in an atmosphere containing 50 mL/L CO_2 and 950 mL/L air. The cells were maintained in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA), 45 mmol/L glucose, 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, USA), 2 mmol/L L-glutamine, and 50 U/mL penicillin and 50 ng/mL streptomycin (Sigma, USA). Induction of adipocytic differentiation of 3T3-L1 cells was performed as described elsewhere[13]. Briefly, 3T3-L1 cells were grown in DMEM supplemented with 10% FBS until confluence. Two days after complete confluence (d 0), cells were cultured in DMEM supplemented with 10% FBS and 0.5 mmol/L 1-methyl-3-isobutylxanthine (Sigma, USA), 0.25 μmol/L dexamethasone (Sigma, USA) and 100 nmol/L insulin (Sigma, USA) for 48 h. From d 2 to 4, the full medium was supplemented with 100 nmol/L insulin only. The cells were then switched back to DMEM containing only 10% FBS for the remaining days. Cultures were replenished every 2 d.

The rat hepatoma cell line H4IIE was cultured at 37℃ in an atmosphere containing 50 mL/L CO_2 and 950 mL/L air, and maintained in DMEM containing 1 g/L glucose and 10% FBS. The cells were incubated in serum-free DMEM (1 g/L glucose) overnight before assay. Glucose levels were adjusted to 4.5 g/L and H4IIE cells were treated with resistin (50 ng/mL) (Alexis, USA) for 2 h prior to insulin (100 nmol/L) stimulation for 2 h. Glycogen synthesis was then assayed as previously described[14].

L6 rat myoblasts were maintained in DMEM supplemented with 10% FBS and differentiated into myotubes by exposure to DMEM supplemented with 2% FBS. Myogenic differentiation to myotubes was confirmed morphologically and biochemically as previously described[15].

Resistin secretion

The supernatants of 3T3-L1 preadipocytes were collected on d 0, 4, 6, and 8 after differentiation and centrifuged to remove cells that might have detached from the culture flasks. The supernatants were kept at -20℃ until assayed for resistin content by enzyme immunoassay (ADL, USA).

RNA preparation and amplification by RT-PCR

Total RNA was isolated from cultured 3T3-L1 cells using the TRIZOL method (Invitrogen, USA). Single strand cDNA synthesis was performed. In brief, the reverse transcription mixture contained 1 μg total RNA, 0.5 μg of oligo d(T) primer, 4 μL of 5 × RT buffer, 0.5 mmol/L deoxynucleotides, 50 U of RNase inhibitor, and 200 U of reverse transcriptase (Promega, USA) in a total volume of 20 μL, the reaction was carried out at 42℃ for 1 h followed by heat inactivation at 95℃ for 5 min. The number of cycles and reaction temperatures used in the PCR assay were optimized to provide a linear relationship between the amount of input template and the amount of PCR product[16]. The primers used for amplification, together with their specific optimum cycling conditions, were as follows:

Mouse resistin (a 415 bp product): [sense primer: 5'-CAA ACAAGACTTCAACTCCC-3', antisense primer: 5'-ACA CACACCCTTCTCCACTA-3', annealing temperature (TA) 58℃, 33 cycles].

β-actin (a 240 bp product): [sense primer: 5'-TAA AGA CCTCTATGCCAACACAGT-3', antisense primer: 5'-CAC GATGGAGGGGCCGGACTCATC-3' annealing temperature (TA) 57℃, 25 cycles].

Glycogen detection

H4IIE cells were serum starved overnight in DMEM containing 0.2% FBS prior to resistin and/or insulin treatment in all experiments. Cells were lysed with 30% KOH and the vials were kept at 100℃ for 20 min. After addition of anhydrous ethanol, the vials were centrifuged at 4000 × *g* for 15 min with the supernatants discarded. Distilled water (0.5 mL) and 1 mL of 0.2% anthrone [0.2 g of anthrone in 100 mL of 98% H_2SO_4 (g/mL), prepared freshly within 1 h] were added, and the vials were placed into boiling water for 20 min. The optical density at 620 nm of the solution in vials was determined by photometry. This method could detect 1.6 μg of glucose per mL, which is equivalent to 1.44 μg of glycogen per mL[17].

2-Deoxyglucose uptake assay

Myotubes were serum starved overnight in DMEM containing 0.2% FBS prior to resistin and/or insulin (10 nmol/L 15 min) treatment in all experiments. Uptake of 2-deoxy-D-[3H] glucose (CIC, China) was assayed for 10 min as previously described[18]. Briefly, the cells were washed with ice-cold phosphate-buffered saline, and then 200 μL NaOH (1 mol/L) was added to each well. Aliquots of the cell lysate were transferred to the scintillation vials for radioactivity counting and the remainder were used for protein assay. Non-specific uptake was determined in the presence of cytochalasin B (10 μmol/L) and subtracted from all values.

Animals

Forty-eight weaned male Sprague-Dawley rats, supplied by the Animal Center of Jiangsu Province, were acclimated to 22℃ in a 12 h light/12 h dark cycle with free access to a standard chow diet for at least a week before grouping. High energy diet contained 10% milk powder, 10% glucose, 10% egg, 10% oil, and 60% standard feed[19]. Animals received this diet for 7 wk. Insulin sensitivity was defined by a value of ISI {ISI = Ln [1/(fasting plasma insulin*glucose)]}[20].

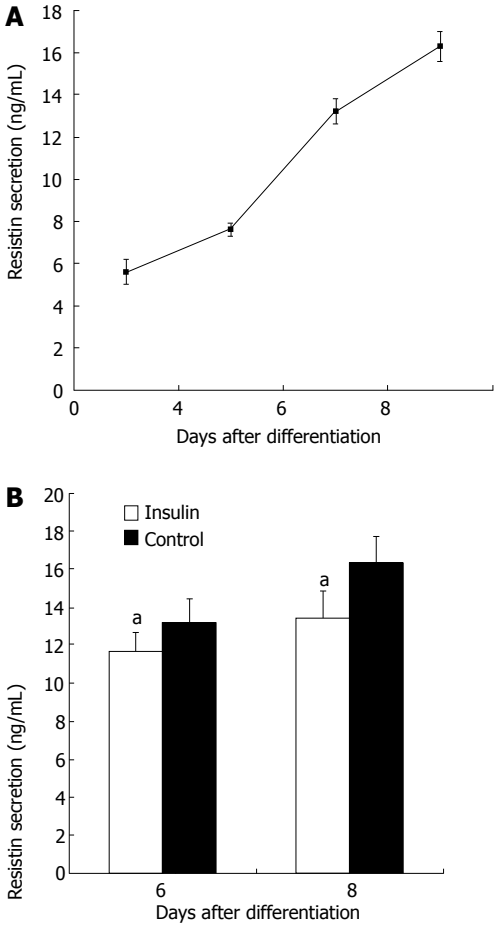


Figure 1 Increased resistin secretion (**A**) and insulin-inhibited resistin secretion (**B**) during 3T3-L1 preadipocyte differentiation. $^{a}P < 0.05$.

Statistical analysis

The data were presented as mean ± SE. Statistical analysis was undertaken using one-way ANOVA or the paired Student's *t*-test where appropriate. Serum resistin levels in diet-induced obese rats were compared with the insulin sensitivity index and levels using the Bivariate correlation. Differences between groups were considered statistically significant when $P < 0.05$.

RESULTS

Increased resistin secretion during 3T3-L1 preadipocyte differentiation inhibited by insulin

Resistin secretion was enhanced during 3T3-L1 preadipocyte differentiation ($P < 0.05$, Figure 1A). Resistin secrtion was about 3-fold higher in matured 3T3-L1 adipoctyes (d 8) than in 3T3-L1 preadipocyte (d 0) ($P < 0.05$). The effect of inslulin (100 nmol/L) on resistin secretion was then assessed in cultured 3T3-L1 adipocytes on d 6 and d 8. Six days after induction of differentiation, 100 nmol/L insulin reduced secretion of resistin by 13% ($P < 0.05$, Figure 1B). Eight days after induction of differentiation, 100 nmol/L insulin reduced secretion of resistin by 20% ($P < 0.05$, Figure 1B).

Upregulation of resistin mRNA level during 3T3-L1 preadipocyte differentiation inhibited by insulin

Resistin mRNA was not detectable in undifferentiated 3T3-L1 cells, but was evident by d 4 after the induction of differentiation into adipocytes (Figure 2A). Resistin mRNA was up-regulated during 3T3-L1 preadipocyte differentiation (Figure 2A), and 100 nmol/L insulin decreased resistin mRNA 6 and 8 d after differentiation (Figure 2B).

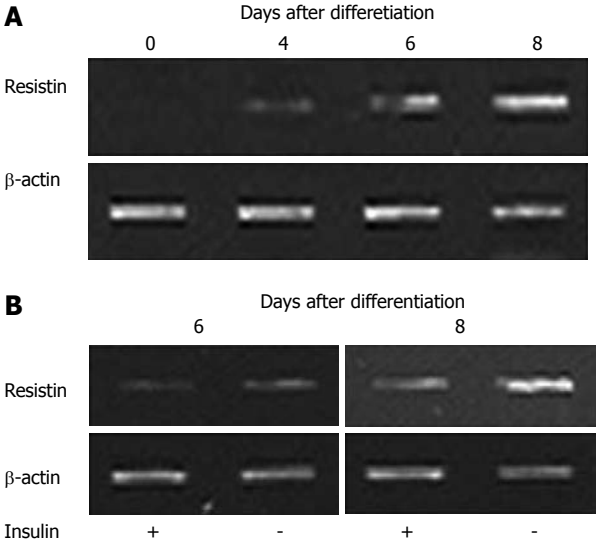


Figure 2 Up-regulation (**A**) and down-regulation (**B**) of resistin mRNA level by insulin during 3T3-L1 preadipocyte differentiation.

Induction of cellular insulin resistance by resistin

Since insulin could inhibit resistin expression and secretion *in vitro*, additional studies were initiated to determine whether resistin plays a role in insulin resistance. Hepatocytes and myotubes are two important targets of insulin[21]. Glycogen synthesis in insulin-stimulated hepatocytes is the most important marker of hepatocyte insulin sensitivity[22]. After treatment with resistin for 2 h, basal glycogen synthesis decreased about 15% and insulin-stimulated glycogen synthesis decreased about 25% in H4IIE cells ($P < 0.05$, Figure 3A). After treatment with resistin for 2 h, basal 2-deoxyglucose uptake decreased about 50% and insulin-stimulated 2-deoxyglucose uptake decreased about 60% in myotubes ($P < 0.05$, Figure 3B), suggesting that resistin could induce cellular insulin resistance, and both hepatocytes and myotubes are important targets of resistin.

Negative correlation of serum resistin with insulin sensitivity but not with serum insulin

Serum resistin levels correlated with rat ISI ($r = -0.662$, $P = 0.005$) in both control and diet-induced obese rats (Figure 4A). Resistin was positively correlated with insulin ($r = 0.592$, $P = 0.016$, Figure 4B), suggesting that insulin could not inhibit resistin secretion *in vivo*.

DISCUSSION

Obesity is associated with insulin resistance and type 2 diabetes, implying that adipose tissue plays a role in the development of such disorders[23]. Besides storing fat, adipose tissue is also an active regulation centre, providing signals to guide metabolism by secreting adipokines[1,2]. Resistin is a newly discovered adipokine that is believed

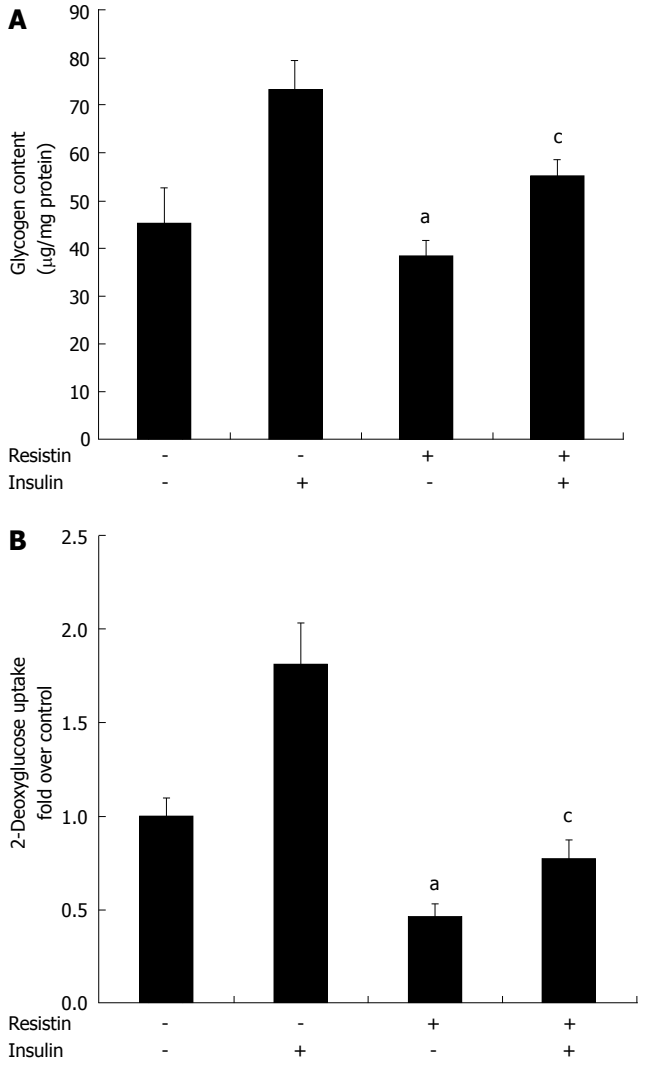


Figure 3 Cellular insulin resistance induced by resistin after treatment of serum-starved H4IIE hepatocytes with 50 ng/mL resistin (**A**) and 100 nmol/L resistin (**B**). $^{a}P < 0.05$ *vs* negative control, $^{c}P < 0.05$ *vs* positive control.

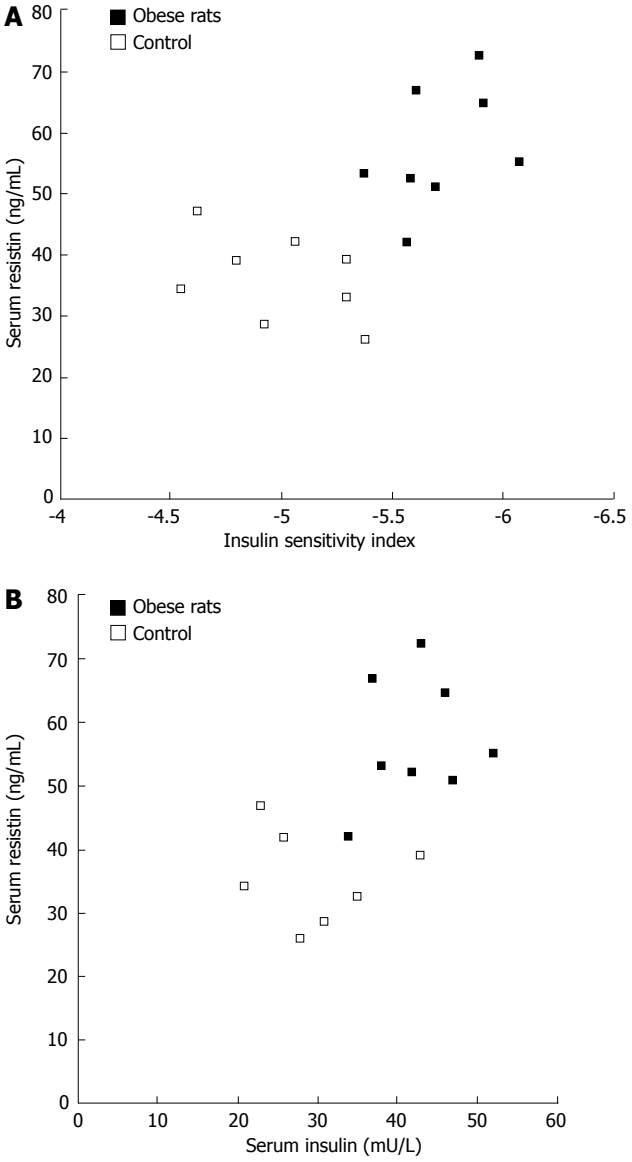


Figure 4 Correlation of serum resistin with rat insulin sensitivity index (**A**) and insulin levels (**B**).

to provide a link between obesity and diabetes[3,4]. Under conditions of insulin resistance and type 2 diabetes, fat tissue is subjected to increased levels of insulin, which may have a major impact on adipokine levels[24,25]. Studies have verified that circulating levels of insulin are correlated with specific adipokines in rodents and humans, with interleukin-6 and leptin levels showing a consistently positive association with insulin levels[24-26]. However, the association of resistin with insulin remains contradictory[27].

Resistin is one of the adipocytokines secreted by adipose tissue and has been shown to modulate both glucose and lipid metabolism *in vivo* and *in vitro*[3,4]. In L6 rat skeletal muscle cells, it has been shown that resistin does not alter insulin receptor signaling but affects insulin-stimulated glucose uptake, presumably by decreasing the intrinsic activity of cell surface glucose transporters[7,8]. In mature 3T3-L1 adipocytes, resistin reduces insulin-stimulated glucose uptake by activating SOCS3, which is an inhibitor of insulin signaling[9]. In addition, it was reported that resistin takes part in insulin resistance in resistin fat-specific transgenic rats by releasing free fatty acids (FFA) from adipose tissue[28].

In the present study, resistin expression and secretion were increased during 3T3-L1 preadipocyte differentiation and resistin mRNA was undetectable in undifferentiated 3T3-L1 cells but was evident by d 4 after the induction of differentiation into adipocytes. The highest expression of resistin mRNA was detected on d 8 after induction of differentiation. Insulin had a marked suppressive effect on resistin mRNA levels in 3T3-L1 adipocytes and inhibited resistin secretion 6 and 8 d after induction of differentiation, suggesting that resistin does not play a role in insulin resistance.

Then, we investigated whether resistin impairs insulin sensitivity *in vitro*, showing that resistin could induce cellular insulin resistance in hepatocytes and myotubes. That is a paradox, because resistin is not regulated by insulin but induces insulin resistance[11]. If both are correct, they will form a deadly insulin-resistin-insulin sensitivity positive feedback loop.

To confirm which one plays the primary role *in vivo*, we analyzed the relationship between serum resistin and

insulin and their sensitivity in diet-induced obese rats. Diet-induced insulin resistance is a relevant model for the most common form of insulin resistance in humans[29]. In our study, serum resistin strongly correlated with rat insulin sensitivity and resistin was positively correlated with insulin, suggesting that insulin could not inhibit resistin secretion *in vivo*. A number of factors can regulate resistin secretion, such as glucose, epinephrine, and somatropin[27,30]. Therefore, insulin may regulate resistin although it is not the major regulator.

In summary, insulin inhibits resistin secretion while resistin induces insulin resistance. Serum resistin correlates with rat insulin sensitivity, meaning that insulin is not the major regulator of resistin. Resistin may play a role in diet-induced insulin resistance by inducing insulin resistance in hepatocytes and myotubes.

COMMENTS

Background

Type 2 diabetes mellitus is closely associated with obesity. Resistin is a recently identified adipokine involved in insulin sensitivity and glucose tolerance. So the investigation of insulin and resistin interaction seems to be important.

Research frontiers

In this article, resistin biological function and interaction of resistin to insulin were studied. We also demonstrated the secretion levels of resistin *in vivo* and *in vitro*.

Innovations and breakthroughs

Resistin induces insulin resistance in hepatocytes, but insulin inhibits resistin secretion *in vitro*. Since serum resistin correlates with rat insulin sensitivity, insulin is not the major regulator of resistin and resistin may play a role in diet-induced insulin resistance by inducing insulin resistance in hepatocytes and myotubes.

Applications

Resistin is a newly identified hormone secreted by adipocytes. Resistin induces insulin resistance both *in vivo* and *in vitro*. This establishes a new field in the pathogenesis of type 2 diabetes.

Peer review

This paper discusses the regulatory mechanisms of resistin and insulin. The study is well designed and the paper is well written. The topic is of scientific value.

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RAPID COMMUNICATION

Lymphangiogenic and angiogenic microvessel density in human primary sporadic colorectal carcinoma

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Abstract

AIM: To investigate the distribution pattern of lymphatic vessels and microvessels in sporadic colorectal carcinoma (SCRC) and their relationship to metastasis and prognosis.

METHODS: The lymphatic vessel density (LVD) and microvessel density (MVD) in tumor tissue obtained from 132 patients with primary SCRC, including 74 with metastases and 58 without metastases, were evaluated by immunohistochemistry using antibodies directed against D2-40 and von Willebrand factor (vWF).

RESULTS: (1) The lymphatic vessels and microvessels at central portions of SCRC often had a reticular architecture with numerous tiny and ill-defined lumina, while those at tumor borders had large and open lumina. The LVD and MVD were both obviously higher in colorectal cancer patients with metastases than in those without ($P < 0.001$). (2) For each one lymphatic vessel increased, there was a 1.45-fold increase in the risk of metastasis in SCRC. The specificity and sensitivity of LVD in predicting metastasis or non-metastasis in SCRC were 71.62% and 56.90%, respectively, and the corresponding LVD was 5. For each one microvessel increased, there was a 1.11-fold increase in the risk of metastasis in SCRC. The specificity and sensitivity of MVD were 66.22% and 51.72%, respectively. (3) Double labeling immunohistochemistry showed D2-40 immunoreactivity to be specific for lymphatic vessels. (4) Univariate analysis indicated that high LVD, high MVD, as well as co-accounting of high LVD and high MVD were associated with patient's poor disease-free survival ($P_{uni} < 0.05$); multivariate analysis indicated that co-accounting of LVD and MVD was an independent prognostic factor of colorectal cancer.

CONCLUSION: D2-40 is a new specific antibody for lymphatic endothelial cells. Lymphogenesis and angiogenesis are commonly seen in SCRC, especially at tumor borders. The detection of LVD and MVD at tumor borders may be useful in predicting metastasis and prognosis in patients with SCRC, and, in particular, co-accounting of LVD and MVD might be a useful prognostic factor in SCRC.

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Key words: Lymphangiogenesis; Angiogenesis; Lymphatic vessel density; Microvessel density; Sporadic colorectal carcinoma; Metastasis

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INTRODUCTION

Lymphangiogenesis (lymph vessel growth) and angiogenesis (blood vessel growth) are critical processes for tumor growth, invasion, and metastasis. Angiogenesis has established its role in the development and progression of a variety of malignancies, playing a crucial role in the dissemination of tumor cells[1,2]. However, lymphatic spread of cancer cells to lymph nodes is an important early event in the metastasis of carcinoma[3]. Previous studies have been limited by the lack of specific lymphatic endothelial makers that allow to discriminate between lymphatics and blood vessels. Recently, the monoclonal antibody D2-40, which is directed against the oncofetal membrane antigen M2A that has been identified in ovarian carcinoma cell lines and germ-cell tumors[4], was reported to be a specific marker for lymphatic endothelium in normal and neoplastic tissue[5,6].

In this study, using the antibody D2-40 and von Willebrand factor (vWF), we investigated the distribution patterns of microvessles and lymphatic vessels and calculated the lymphatic vessel density (LVD) and

microvessel density (MVD) in sporadic colorectal carcinoma (SCRC). Moreover, we analyzed their relationship with tumor metastasis and disease prognosis. By double labeling immunohistochemistry we were able to confirm D2-40 specificity.

MATERIALS AND METHODS

Patients and specimens

A total of 132 colorectal carcinoma samples were obtained from the archives of the Department of Pathology, Cancer Hospital, Fudan University, from July 2004 to May 2005. Samples were derived from patients who were solely surgically treated without prior chemo- or radiotherapy. There were 77 men (58.3%) and 55 women (41.7%) with a median age of 57 years (range, 22-82 years), 46 patients with colon and 86 patients with rectum tumors. 74 patients (56.1%) had histologically confirmed lymph node metastases, whereas the remaining 58 patients (43.9%) were found to have no clinical or histopathologic evidence of lymph node involvement. According to the current World Health Organization classification, tumors of 14 (10.6%) patients were well differentiated, that of 96 patients (72.7%) were moderately differentiated and that of 22 (16.7%) patients were poorly differentiated. Follow-up for this cohort was updated to September 2006. Complete data were available for 127 (96.2%) patients, with a median follow-up time of 22 mo (range, 2-26 mo). At the end of follow-up, 18 (14.17%) patients had died of disease and 9 (7.09%) had developed distant metastases. The study was approved by the local ethical committee in Cancer Hospital of Fudan University.

Immunohistochemistry

Tissues were fixed in 10% buffered formalin, processed, and stained with hematoxylin and eosin (H&E). H&E-stained slides of all samples were reviewed to confirm the diagnosis. One paraffin block with the bulk of tumor tissue was used for immunohistochemical studies. All slides showed the nonneoplastic colorectal tissue-carcinoma junction. Sections, 3 mm-thick, of formalin-fixed paraffin embedded tissues were cut and mounted on coated slides. The sections were deparaffinized in xylene and rehydrated in a descending ethanol series. Heat induced epitope retrieval techniques were used for antigen retrieval as follows: citrate buffer (pH 6.0) and a water bath at 95℃-98℃ for 30 min for D2-40, Tris-EDTA buffer (pH 8.0) and a water bath at 95℃-98℃ for 30 min for vWF. Sections were incubated for 10 min in 3% hydrogen peroxide to quench endogenous tissue peroxidase. The sections were immunostained with a monoclonal antibody (Clone D2-40, m3619; Dako Corp., Carpinteria, CA, USA) at a 1:100 dilution directed against D2-40 and a rabbit polyclonal antibody (A0082; Dako) at a dilution of 1:200 directed against vWF. Tissue sections were incubated with the primary antibody for 12 h at 4℃. After washing with phosphate-buffered saline, a Super picture secondary antibody (Zymed Lab Inc, CA, USA) and 3-3' diaminobenzidine detection kit (Dako) were used. A lymphangioma tissue sample served as a positive control and replacement of the primary antibody by PBS as a negative control. Thirty samples were picked randomly for double labeling immunohistochemistry (Histostain™-DS double labeling immunohistochemistry kit, Zymed). The sections were first subjected to D2-40 staining using BCIP/NBT as chromogenic agent, followed by a vWF staining using AEC as chromogenic agent.

Evaluation of immunostaining and vessels counting

Immunohistochemical reactions for D2-40 and vWF were interpreted independently by two authors (Y.G. and Z.GH.) using a two-headed microscope. After scanning the immunostained section at low magnification (× 100), five areas of carcinoma with the greatest number of distinctly highlighted intratumoral lymphatic/vascular foci (hot spots) were selected and vessels were counted in a representative high magnification (× 200) field in each of these five areas. Single immunoreactive endothelial cells, or endothelial cell clusters separate from other microvessels, were counted as individual microvessels. Endothelial staining in large vessels with tunica media, and non-specific staining of nonendothelial structures, were disregarded in microvessel counts. Mean visual microvessel density for D2-40 and vWF was calculated as the average of five counts[7]. In double labeling immunohistochemistry, lymphatic vessels were amethyst and blood vessels were bright red.

Statistical analysis

Mean differences in microvessel counts were compared with the use of "*t*" tests. The probability of differences between the high-vessel group and the low-vessel group in disease-free survival (DFS) was determined as a function of time by the use of Kaplan-Meier test, with significance probing by applying the log-rank test. We used multivariate regression analysis based on the Cox proportional hazard model to test the independence of these parameters to predict overall survival. Logistic regression analysis and ROC curve were used to determine specificity and sensitivity of LVD and MVD in assessment of metastasis in SCRC. Generally, P value < 0.05 was regarded as significant. For all statistical procedures, SPSS v12.0 and Stata v7.0 software were used.

RESULTS

Expression pattern of lymphatic vessels in SCRC and correlation of LVD with clinicopathologic parameters

A tissue sample of lymphangioma was chosen as a positive control for D2-40 staining. As shown in Figure 1A, endothelial cells in this sample were found to be positive. In SCRC samples, definite lymphatic vessels were stained by D2-40. Lymphatic vessels at central portions were rare, even absent in some case, and often had a reticular architecture with numerous tiny and ill-defined lumina (Figure 1B). The LVD was higher at tumor borders (10.32 ± 4.94) than at central portions and had large and open lumina (Figure 1C). The LVD was obviously higher in the CRC samples with metastases (12.08 ± 4.96) than in those without (8.26 ± 4.08) ($P < 0.001$). There was no significant correlation between

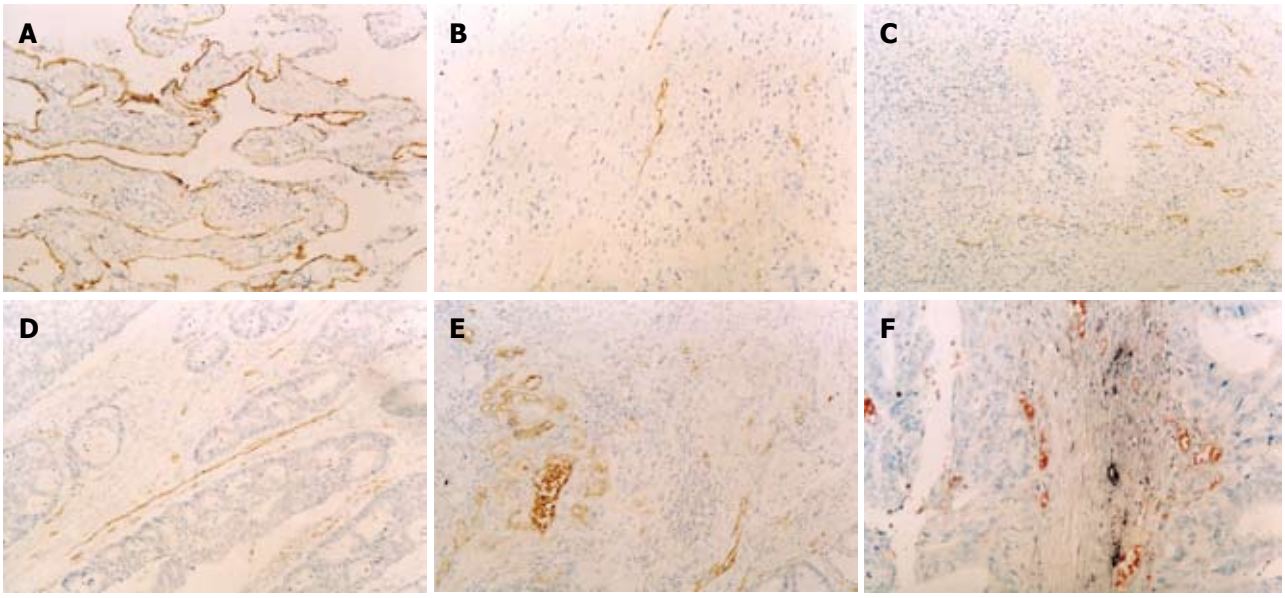


Figure 1 Immunohistochemical stainings of D2-40 (**A**, **B**, **C** × 100), vWF (**D**, **E** × 100) and double labeling immunohistochemistry (**F** × 200, red: Blood vessels labled by vWF; amethyst: Lymphatic vessels by D2-40). **A**: Lymphangioma (positive control); **B-F**: Colorectal carcinoma.

Table 1 Correlation of LVD/MVD with clinicopathologic parameters of SCRC

Clinico-pathologic features	*n*	LVD		MVD	
		mean ± SD	*P*	mean ± SD	*P*
Age (yr)					
<50	28	11.03 ± 5.71	0.396	19.58 ± 9.56	0.332
⩾ 50	104	10.14 ± 4.73		21.92 ± 11.76	
Gender					
Male	77	10.50 ± 4.73	0.641	21.00 ± 11.74	0.609
Female	55	10.09 ± 5.27		22.02 ± 10.82	
Tumor size					
< 5 cm	77	10.37 ± 4.84	0.904	22.26 ± 11.92	0.313
⩾ 5 cm	55	10.26 ± 5.13		20.24 ± 10.46	
Location					
Right colon	23	9.36 ± 3.95	0.323	23.36 ± 10.33	0.248
Left colon	23	9.52 ± 4.65		18.06 ± 11.25	
Rectum	86	10.80 ± 5.23		21.81 ± 11.55	
Invasive depth					
Intra-deep muscular	36	9.31 ± 5.82	0.15	21.74 ± 11.02	0.845
Whole layer	96	10.71 ± 5.82		21.30 ± 5.76	
Degree of differentiation					
Well	14	8.38 ± 4.60	0.119	21.28 ± 9.38	0.962
Moderately & poorly	118	10.56 ± 4.95		21.44 ± 11.58	
Lymph node metastasis					
Yes	74	12.01 ± 4.90	< 0.001	24.00 ± 11.98	0.003
No	58	8.18 ± 4.12		18.14 ± 9.58	

LVD: Lymphatic vessel density; MVD: Microvessel density.

LVD with age, gender, tumor size, location, degree of differentiation, or invasive depth ($P > 0.05$) (Table 1).

Logistic regression analysis and ROC curve of LVD in SCRC

A logistic model was built with LVD as an independent and metastasis as dependent variable (Prob > chi2 = 0.0000, Pseudo R2-0.1176). According to the analysis for of the logistic model, we found an OR value of 1.45, meaning that for each lymphatic vessel there is an 1.45-fold increase in the risk of metastasis in SCRC. The specificity and sensitivity of LVD in predicting metastasis or non-metastasis in SCRC were 71.62% and 56.90%, respectively, and the corresponding LVD was 5 (Figure 2A).

Expression pattern of blood microvessels in SCRC and correlation of MVD with clinicopathologic parameters

There were abundant microvessels labeled by vWF in SCRC, with the similar distribution pattern to lymphatic vessels. The distribution feature and quantity of microvessels were different within one tumor sample. That is, microvessels at surrounding part of tumor were abundant (mean = 21.24 ± 11.98) and had large and open lumina (Figure 1D). But, microvessels at central portions were seldom and often had a reticular architecture with numerous tiny and ill-defined lumina (Figure 1E). The MVD was obviously higher in the colorectal carcinoma samples with metastasis (23.74 ± 12.02) than in those without (18.00 ± 9.44) ($P = 0.003$). There was no significant correlation between MVD with age, gender, tumor size, location, degree of differentiation, or invasive depth ($P > 0.05$) (Table 1).

The specificity of D2-40 for lymphatic vessels and vWF for microvessels was confirmed by double labeling immunohistochemistry. There was no cross-reaction between the two antibodies. Lymphatic vessels and microvessels distributed separately in tumor borders without obvious relationship (Figure 1F).

Logistic regression analysis and ROC curve of MVD in SCRC

A l-Logistic model with MVD as an independent and metastasis as the dependent variable could be established (Prob > chi2 = 0.0022, Pseudo R2 = 0.0519). According to this model, we found an OR of 1.11, meaning that for each microvessel there is an 1.11-fold increase in the risk of metastasis in SCRC. According to ROC curve analysis, the specificity and sensitivity of MVD in predicting metastasis or non-metastasis in SCRC were 71.62% and 56.90%, respectively (Figure 2B).

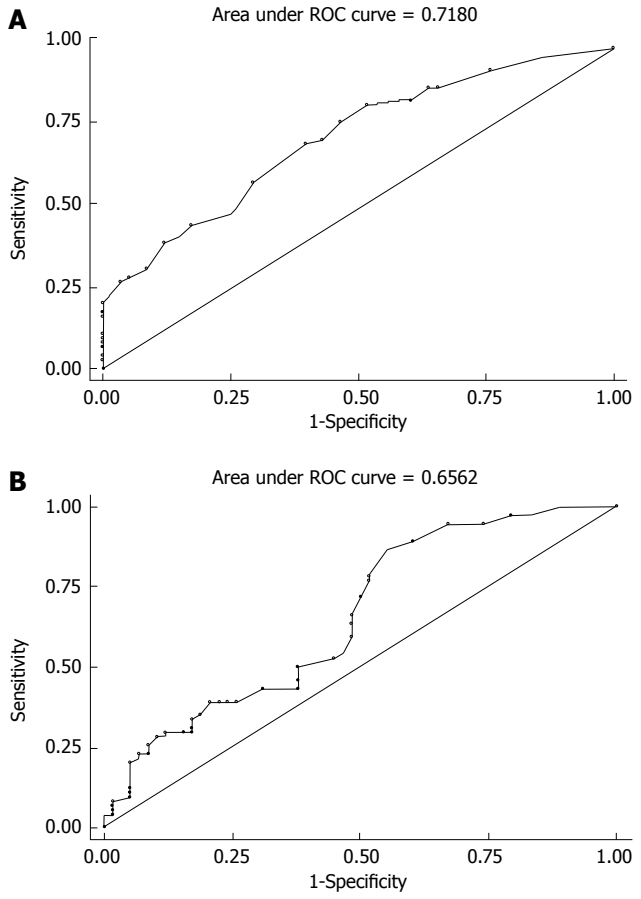


Figure 2 ROC curve of LVD (**A**) and MVD (**B**).

Univariate survival analysis

LVD = 5 was defined as demarcation value according to the ROC curve of LVD. LVD > 5 was high LVD group and LVD < 5 was low LVD group. The median of MVD (median = 9.33) was defined as demarcation value. MVD > 9.33 was high MVD group and MVD < 9.33 was low MVD group. LVD > 5 and MVD > 9.33 was co-high group. In univariate analysis with Kaplan-Meier for disease-free survival (DFS), high LVD (P = 0.0303), high MVD (P = 0.0196), co-high LVD and MVD (P < 0.0001) were associated with patient's poor DFS ($Puni$ < 0.05) (Figure 3).

Multivariate survival analysis

All factors were brought into a Cox regression model, including some clinico-pathologic parameters such as patients' age, gender, tumor size, location, degree of differentiation, invasive depth and metastasis, LVD, MVD, and co-accounting of LVD and MVD. Multivariate analysis indicated that besides metastasis (P = 0.004), gender (P = 0.012), and location (P = 0.028), co-accounting of LVD and MVD was an independent prognostic factor of colorectal carcinoma (P = 0.014).

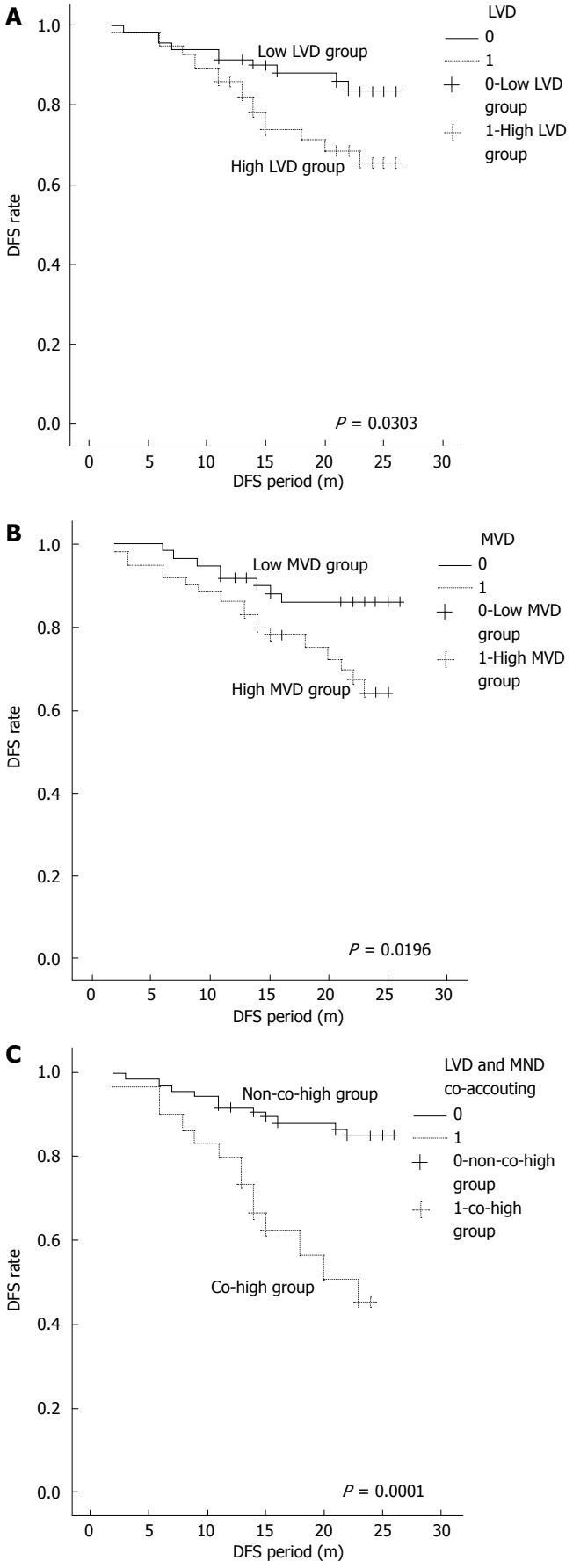


Figure 3 Survival curve of LVD (**A**), MVD (**B**) and co-accounting of LVD and MVD (**C**).

DISCUSSION

This study is one of the first attempts to quantify colorectal carcinoma lymphangiogenesis and angiogenesis in the same sample by microvessel density using the novel lymphatic marker D2-40 and vWF. We compared the distribution pattern and density of lymphatic vessels and

blood vessels, and related the results to clinicopathologic parameters and the outcome of colorectal carcinoma. Moreover, specificity of the two antibodies was confirmed by double labeling immunohistochemistry.

Significance of lymphangiogenesis in SCRC

The lymphatic system is the primary pathway of metastasis for most human cancers. Lymphangiogenesis refers to the development and proliferation of new lymphatics from host vessels. Recently, antibodies specific for lymphatic endothelium have become available, providing important new insights into the process of tumor-associated lymphangiogenesis and its possible clinical relevance. Many studies have reported that tumors are able not only to induce lymphangiogenesis, but also to enhance lymphatic metastasis[8,9].

There are some antibodies for lymphatic endothelium now, including VEGFR-3/flt4 (vascular endothelial growth factor receptor 3/fms-like tyrosine kinase 4)[10], LYVE-1 (lymphatic endothelial hyaluronan receptor)[11], Prox-1[12], CD31[13], and podoplanin[14]. VEGFR-3, the receptor for vascular endothelial growth factors (VEGFs) C and D, is expressed on lymphatic endothelium and may play a role in lymphangiogenesis[15,16]. But, some studies indicated that VEGFR-3 was also involved in blood vessel angiogenesis in the adult and it was not a specific antibody for lymphatic endothelium[17]. CD31 also stained both in blood vessel and in lymphatic vessel. But, the monoclonal antibody D2-40 is a highly selective marker of lymphatic endothelium in sections of both frozen and formalin-fixed paraffin-embedded normal and neoplastic tissues. In a direct comparison of D2-40 and CD31 on paraffin sections of a series of tumors derived from lymphatic endothelium (lymphangiomas) and blood vessel endothelium (hemangiomas), D2-40 stained all lymphagiomas (10/10) but no hemangiomas (0/10). Conversely, CD31 stained a fraction of lymphangiomas (5/10) but all hemangiomas (10/10)[13].

Pusztaszeri *et al*[18] found D2-40 to be a sensitive and relatively specific marker for lymphatic endothelium in all parenchymatous organs. It stained lymphatic endothelium only and it has been demonstrated to recognize tumor-associated lymphatic vessels in many tumors[19,20]. Lymphatic vessels stained by D2-40 in our study often had a defined lumen with thin walls lacking erythrocytes, which were all the features of lymphatic vessels. There was no cross-reaction between D2-40 for lymphatic vessels and vWF for microvessels in double labeling immunohistochemistry. D2-40 is specific for lymphatic vessels[21,22].

We also found lymphatic vessels to show a different distribution between tumor central portions and tumor borders in SCRC. Lymphatic vessels at central portions were seldom, even absent in some cases, but abundant at tumor borders. This phenomenon may be due to the inside pressure of the tumor. The LVD was obviously higher in the cases of colorectal carcinoma with metastasis than that in cases without, indicating that tumor cells might migrate from a primary site to lymph nodes through lymphatic vessels at the tumor borders. The increase of LVD was related to lymph nodes metastasis in SCRC. According to the logistic model and ROC curve analysis, we found an OR of 1.45, meaning that for each lymphatic vessel there is a 1.45-fold increase in the risk of metastasis in SCRC. The specificity and sensitivity of LVD in predicting metastasis or non-metastasis in SCRC were 71.62% and 56.90%, respectively, and the corresponding LVD was 5. The univariate analysis showed high LVD to be associated with patient's poor DFS. Thus, LVD was an important factor to predict metastasis and prognosis for the patients with SCRC. Lymphatic vessels were composed of a single, non-fenestrated endothelial layer with wide and irregular lumina. Endothelial cells, with scant cytoplasm were often overlapping. But there were infrequent interendothelial tight junctions and no basement membrane and encircling pericytes on them. The morphology of lymphatic vessels differed from that of blood microvessels consequently made it was easy for fluid, particles, and tumor cells to pass into the lymphatic vessels. The surface area between tumor cells and lymphatic endothelial cells increased with the enhancement of LVD, which promoted the migration of tumor cells to lymph nodes[2]. So lymphangiogenesis is associated with an increased incidence of lymph nodes metastasis, and it is possible that this step is essential to the metastatic process. Recently, some studies on gastric cancer[23] and breast cancer[24,25] demonstrated LVD to be correlated with lymph nodes metastasis.

Significance of angiogenesis in SCRC

Angiogenesis, the formation of new blood vessels from the endothelium of the existing vasculature, is fundamental in tumor growth, progression and metastasis, especially for colorectal carcinoma[26]. The complex network of tumor blood microvessels guarantees adequate supply of tumor cells with nutrients and oxygen and provides efficient drainage of metabolites. In addition to primary tumor growth, metastatic tumor growth depends upon neovascularization in at least two steps: First, malignant cells must exit from a primary tumor into the blood circulation after the tumor becomes neovascularized. Second, after arrival at distant organs, metastatic cells must again induce angiogenesis for a tumor to expand to a detectable size[27,28]. Zhao *et al*[29] suggests MVD as one of the important prognostic factors for gastric cancer patients by immunohistochemical staining of endothelial protein factor Ⅷ-related antigen. Romani *et al*[30] evaluated retrospectively the effect of CD105-assessed (a marker of neovascularization in solid malignancies) angiogenesis on the risk of developing metastatic disease in colorectal cancer. One hundred and twenty-five paraffin-embedded samples were analyzed by immunohistochemical methods using CD105 monoclonal antibody. The CD105-vessel count was found to be strongly correlated with the occurrence of metastatic disease. The median CD105-positive vessels in patients with and without metastatic disease were 24.7 and 13.2 vessels/mm^2, respectively ($P < 0.001$). For each one microvessel increase in the vessels count per 400 × field, there was a 1.42-fold increase in the risk of metastatic disease ($P < 0.001$). We found the distribution feature and quantity of microvessels were different within one tumor sample labeled by vWF, which is similar to the distribution pattern of lymphatic vessels.

That is, microvessels at surrounding part of tumor were abundant (mean = 21.24 ± 11.98) and had large and open lumina. In contrast, microvessels at central portions were less frequent and often had a reticular architecture with tiny and ill-defined lumina. The MVD was obviously higher in samples from colorectal carcinoma patients with metastasis than in those without. Our results indicated metastasis of SCRC to be associated with MVD. According to the logistic model, we found an OR of 1.11, meaning for each microvessel there is an estimated 1.11-fold higher risk of metastasis in SCRC. According to ROC curve analysis, the specificity and sensitivity of MVD in predicting metastasis or non-metastasis in SCRC were 71.62% and 56.90%, respectively. The univariate analysis showed high MVD to be associated with patient's poor DFS. The lymphatic system was the most common pathway of metastasis for SCRC, accounting 60%. And the second primary pathway was blood vessels. Compared with LVD, the specificity and sensitivity of MVD in predicting metastasis or non-metastasis was lower. But MVD still played a role in roughly predicting prognosis of patients with SCRC.

Lymphangiogenesis and angiogenesis are essential for metastasis of tumor cells. We evaluated the specificity (71.62% *vs* 66.22%) and sensitivity (56.90% *vs* 51.72%) of LVD and MVD in SCRC. The specificity and sensitivity of LVD was slightly higher than that of MVD. Thus, neither LVD nor MVD alone were sufficiently specific and sensitive to predict metastasis or prognosis. We thus combined LVD with MVD to co-account. Univariate analysis indicated that co-accounting of high LVD and high MVD were closely associated with patient's poor DFS and multivariate analysis indicated besides metastasis, gender and location, co-accounting of LVD and MVD to be independently predictive. Thus, evaluating lymphangiogenesis and angiogenesis are thought to be clinically important, particularly for the estimation of the metastatic risk and prognosis.

In summary, D2-40 is a new specific antibody for lymphatic endothelial cells. Lymphogenesis and angiogenesis are commonly seen in SCRC, especially at tumor borders. The detection of LVD and MVD at tumor borders may be useful in predicting metastasis and prognosis in patients with SCRC, and especially, the co-accounting of LVD and MVD might be used as a prognostic factor of SCRC.

ACKNOWLEDGMENTS

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COMMENTS

Background

More and more researchers focus on the importance of lymphogenesis in tumorigenesis and metastasis because of the development and update of new markers for lymphatic vessels. It is a common phenomenon that metastatic local lymph node in sporadic colorectal carcinoma (SCRC), but the relationship between the lymphogenesis and metastasis is not clear.

Research frontiers

D2-40 is a new specific marker for lymphatic endothelium in normal and neoplastic tissue. Recent studies demonstrated D2-40 to be probably useful for diagnosis of malignant mesothelioma and research on lymphatic spread of cancer cells.

Innovations and breakthroughs

We investigated the role of lymphogenesis and angiogenesis using D2-40 and their distribution patterns in Sporadic colorectal carcinoma (SCRC). Moreover, we assessed how to predict metastasis by the increase of Microvessel density (MVD) and Lymphatic vessel density (LVD) and determined specificity and sensitivity.

Applications

Our study found LVD and MVD to be related to prognosis of SCRC. We calculated the risk ratio in predicting metastasis in SCRC by statistical procedures. In the future, we will be able to give an estimate on the prognosis and the survival of patients with colorectal carcinoma by accounting LVD and MVD.

Terminology

SCRC: Sporadic colorectal carcinoma; LVD: Lymphatic vessel density; MVD: Microvessel density.

Peer review

The results of this paper are reliably to the conclusions. The innovative and significant points conform to the background, objectives, materials and methods, results and conclusions. There is no conflict of interest, nor ethics problems. This is significant research.

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RAPID COMMUNICATION

Expression of phosphatase and tensin homolog deleted on chromosome ten in liver of athymic mice with hepatocellular carcinoma and the effect of Fuzheng Jiedu Decoction

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Abstract

AIM: To explore the expression of phosphatase and tensin homolog deleted on chromosome ten (PTEN) in liver of athymic mice with hepatocellular carcinoma (HCC) and the effect of Fuzheng Jiedu Decoction (FJD).

METHODS: Forty eight male BALB/c athymic mice models were built by Bel-7402 with an indirect method. After 24 h of postoperation, the 48 athymic mice were distributed randomly into 4 groups: A, B, C, D, each group had 12 athymic mice. Group A were treated by intragastric administration with FT207 (Tegafur) for 4 wk. Group B, C and D were treated by intragastric administration with FJD (complex prescription of Chinese crude drug) that had been delegated into 3 kinds of density as the low, middle, and high for 4 wk. At last, athymic mice were put to death, live time, volume of tumors, exponent of tumors and the tumor metastasis in livers were observed; and PTEN was detected in hepatic tissue, latero-cancer tissue and cancer tissue by immunohistochemistry.

RESULTS: Four weeks later, the total survival rate in treatment group (A + B + C) was 50% and higher than the control group (0%) treated by FT207, ($P < 0.01$). The survival rate in group A, B, C was higher than in group D, and except group A with D, there was significant differentces (Fisher's Exact Test $P = 0.05$ or 0.01). And no differences were observed between the treatment groups and the control group in volume of tumors and exponent of tumors ($P > 0.05$). Tumor metastasis in livers of the treatment group was less than the controls (Fisher's Exact Test, $P = 0.021$). The result of immunohistochemistry showed that the intensity of PTEN in latero-cancer tissue was the highest, and then the hepatic tissue, the lowest was cancer tissue (Kruskal-Wallis test, $\chi^2 = 60.67$, $P = 0.000$). It also showed that the intensity of PTEN in treatment groups (A, B, C) was higher than the control group (D) ($F = 5.90$, $P = 0.002$ in hepatic tissue and $F = 15.99$, $P = 0.000$ in latero-cancer tissue and $\chi^2 = 26.08$, $P = 0.000$ in cancer tissue), and group B is the highest in the treatment groups ($P < 0.05$, $r = 0.01$. respectively). However, there was no significant statistic difference between group A and group C ($P > 0.05$).

CONCLUSION: FJD can prolong the survival time and decrease tumor metastasis in livers of these experimental mice. Mechanisms of FJD healing HCC may partially be explained by enhancing the expression of PTEN in liver.

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Key words: Phosphatase and tensin homolog deleted on chromosome ten; Athymic mice; Hepatocellular carcinoma; Fuzheng Jiedu Decoction

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major cancer killers. Although surgical resection, liver transplantation and percutaneous ablation are considered as effective treatment for HCC[1-3], Traditional Chinese Medicine (TCM) has been widely used as combined therapies in treating the disease in China. According to the theory of TCM, the main mechanism of HCC

is deficiency of vital qi and exuberant pathogens, thus strengthening body resistance and disintoxication is the major method of treating HCC[4]. The previous study demonstrated that reduced expression levels of PTEN are involved in the pathogenesis of HCC. Moreover, decreased phosphatase and tensin homolog deleted on chromosome ten (PTEN) expression was correlated with tumor progression and poor prognosis in patients with HCC[5-7], whether TCM can down-regulate the expression of PTEN in HCC is still unknown. The aim of our study is to explore the effect of Fuzheng Jiedu Decoction (FJD), complex prescription of Chinese crude drug in treating BALB/c athymic mice with HCC, as well as the expression of PTEN. We proclaim that the animal study was acknowledged by the Ethical Committee of The First Affiliated Hospital of Sun Yat-Sen University committee in the materials and methods section. The results are reported as following.

MATERIALS AND METHODS

Study animals and environmental conditions

Forty eight BALB/c athymic male mice, 4-6 wk, were purchased from The Experimental Animal Center of Traditional Chinese Medicine university of Guangzhou. The animals were housed individually in cages and kept in a room maintained at a temperature of 23 with a relative humidity (RT) of 55 with a 12-h/12-h light/dark cycle. Solid rodent chow and tap water were given ad libitum.

Drugs and reagents

FJD (Application: 200710026976.6) consists of the following indegredients: Ezhu (Rhizoma Curcumae) 15 g, Banzhilian (Herba Scutellariae Darbatae) 30 g, *etc*. They were decocted routinely and then made into a final concentration of 2 g/mL which extracted and prepared by college of pharmacy of Traditional Chinese Medicine University of Guangzhou. The prepared herbal pieces were purchased from Guangzhou City Pharmacy Company and consistent with the requirement of Pharmacopoeia of the People's Republic of China[8]; FT207 parenteral solution (Tegafur parenteral solution), 5 mL/0.2 g per ramus, Shandongqilu production (production batch number 06020032); rabbit anti-human PTEN polyclonal antibody (production batch number 60182150), Power Vision™ Two-Step Histostaining Reagent (production batch number 125135), were bought from Beijing Zhongshan Jinqiao biotechnology limited company.

Preparation of animal model

The athymic mice with indirect orthotopic transplantation tumor models were established in accordance with the method by Dr. Zheng Jianhua[9]. The animals were inoculated Bel-7402 hepatoma carcinoma cell with concentration of 1 million cells/mL on their waist and back, until subcutaneous transplantation tumor grew to diameter 1 cm, then cut down the tumor. Remove necrotic tissue in the tumor and cut into pieces about 1 mm^3 in Hanks liquid. Anesthetize the athymic mice in abdominal cavity with Pentobarbital 45 mg/kg weight, transverse incise the left upper quadrant, expose liver, take 1 piece of tumor tissue, use bodkin pinhead (20° angle of slope, deep 3 mm) imbed the tumor tissue in deep part of hepatic lobes parenchyma of athymic mouse in *ex vivo* 40 min, compresse the incision to stop bleeding then close abdomen.

Grouping and the treatment of the animals

Athymic mice were raised in SPF condition in divided cages. After 24 h of postoperation, the 48 athymic mice were distributed randomly into 4 groups, every group has 12 mice. Low concentration group (A): drench the Ganaifang (0.25 g Chinese crude drug/mL, 0.2 mL/10 g weight) with the dose of 10 times human unit kilogram weight. Middle concentration group (B): drench the Ganaifang (0.5 g Chinese crude drug/mL, 0.2 mL/10 g weight) with the dose of 20 times human unit kilogram weight. High concentration group (C): drench the Ganaifang (1.0 g Chinese crude drug/mL, 0.2 mL/10 g weight) with the dose of 40 times human unit kilogram body weight. Chemotherapy group (D): drench the FT207 parenteral solution (Tegafur parenteral solution) (8 mg/mL, 0.2 mL/10 g weight) with the dose of 5 times human per day.

Record live time of each athymic mouse in the process of observation. When the treatment was ended, get blood from athymic mice eyeball, and put them to death, then dissect them, meanwhile record the tumor volume, tumor index number (the weight of the tumor/the weight of the mouse), fix the normal hepatic tissue, latero-cancer tissue and cancer tissue in 4% neutral formalin, sent to pathology laboratory to make paraffin imbedding microtome sections (4 mm thick serial sections) fixed in silicification glass.

Pathologic detection

Histologic detection: dying with the routine hematoxilin -eosine (HE).

Immunohistochemistry detection: 4 μm thick paraffin sections were baked at 65℃ until get deparaffinage and hydration; incubate in 3% Hydrogen Dioxide about 5-10 min to inactivate the endogenous peroxidase; microwave repairs the antigen; sealed by 10% normal goat serum; PTFN multiclone rabbit antibody (1:100) was incubated overnight at 4℃, washed by PBS 2 min for 3 times; dropwise goat anti-rabbit IgG antibody-HRP multimer, incubated about 30 min at 37℃, washed by PBS, 2 min × 3 times; DAB coloration; washed thoroughly by distilled water. In the same time, PBS was used as first-antibody and second-antibody in negative control.

Evalution of coloration result: using Bresalier[10] semiquantitative formula to judge coloration result. Selecting randomly 10 fields of vision from every section when enlarged 200 times, then we classified the cell coloration intensity into four categories: Negative, cell hasn't colored (0); Cell has colored buff (1); claybank (2); chocolatebrown (3). Count the number of field of vision of each intensity, and according to the formula calculate the average coloration intensity. IS (intensity score) = $\sum[(0 \times F0) + (1 \times F1) + (2 \times F2) + (3 \times F3)]$, $F = n/10$ (n = 0, 1, 2, 3 the number of field of vision of different score). Two people read the section two times with double

Table 1 Survival time in different groups

Group	*n*	Survival days mean ± SD	Survival rate (%) 3 wk	4 wk
A	12	23.67 ± 4.92	10/12 (83.3)	5/12 (41.7)
B	12	26.08 ± 2.31	12/12 (100)[a]	6/12 (50)[a]
C	12	25.92 ± 2.71	12/12 (100)[a]	7/12 (58.3)[b]
D	12	18.83 ± 2.29	7/12 (58.3)	0/12 (0)

[a]$P < 0.05$, [b]$P < 0.01$ *vs* group D.

Table 2 Volume and index of tumors (mean ± SD)

Group	*n*	Volume (mm^3)	Index
A	12	466.43 ± 645.66	0.0037 ± 0.0403
B	12	215.91 ± 275.23	0.0305 ± 0.0403
C	12	325.23 ± 464.30	0.0313 ± 0.0436
D	12	309.7 ± 309.72	0.0462 ± 0.0296

Table 3 Tumor metastasis in livers

Group	Rate of tumor metastasis in liver (%)
A + B + C	23/36 (63.9)[a]
A	7/12 (58.3)[c]
B	9/12 (75)
C	7/12(58.3)[c]
D	12/12(100)

[a]$P < 0.05$ *vs* group D; [c]$P < 0.05$ *vs* group D.

blind method, calculate IS for each time, and get the two times average as the result.

Statistical analysis

Using SPSS 14.0 statistic software, variance analysis and rank-sum test were used for measurement data; chi square test and rank-sum test were used for numeration data.

RESULTS

General situation

The dissection witness that achievement ratio of making model is 100%; at the end of the experiment, get 48 utility pathologic samples. Most tumors were enormous, some were accompanied multilesions in the liver and/or lung.

It was demonstrated that the model was stable and facility, consistent with dissection characteristic of human liver cancer.

Survival state

After 3 wk of drug intervention, the total survival rate in treatment group (A + B + C) was 94.4% and much higher than the chemotherapy group (58.3%) ($P = 0.007$, Fisher's Exact Test). Though the survival rate in group A was higher than that of group D, no differences were observed between them ($P > 0.05$). The survival rate in group Band C is higher than in group D, there was a significant difference ($P = 0.014$). 4 wk later, the total survival rate in treatment group (A + B + C) was 50% and much higher than the chemotherapy group (0%) ($P = 0.02$). Though the survival rate in group A was higher than that of group D, no difference were observed between them ($P > 0.05$). The survival rate in group B and C was higher than in group D, there was significant difference, ($P < 0.05$ or 0.01) (Table 1).

Table 4 Expression intensity of PTEN in the 3 kinds of tissue ($\alpha = 0.01$)

Kind of tissue	*n*	PTEN intensity median
Normal hepatic tissue	48	1.00
Latero-cancer tissue	48	1.31
Cancer tissue	48	0.23

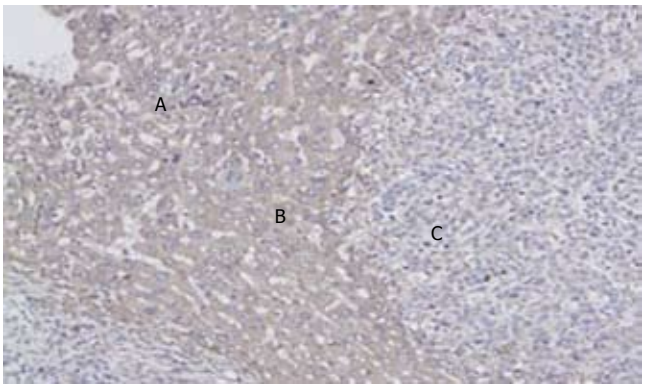


Figure 1 Intensity of PTEN: Latero-cancer tissue. A: > cancer tissue; B: > hepatic tissue; C: Two-Step, × 100.

Tumor state

No differences was observed between the group A, group B, group C and the group D in volume and index of tumors ($P > 0.05$) (Table 2).

The state of tumor metastasis in liver

After the transplantation tumors in liver of each mouse was taken out, then the remain liver were observe by microscope, if it existed hepatoma carcinoma cell, We diagnosed the liver had the metastases. Tumor metastasis in livers of group A and group C were both 58.3% (7/12) and much less than the group D 100% (12/12) $P = 0.037$. Tumor metastasis in livers of group A, group B and group C was 63.9% (23/36) and much less than the group D ($P = 0.021$), but there was no difference among group A, group B and group C (Table 3).

The expression intensity of PTEN in different tissues

Three groups were compared with each other applied Kruskal-Wallis test, $\chi^2 = 60.67$, $P = 0.000$. The result showed that the intensity of PTEN in latero-cancer tissue was the highest, and then the normal hepatic tissue, the lowest was cancer tissue (Table 4, Figures 1 and 2).

Interclass analyses positive expression intensity of PTEN in three kinds tissue

Tested by One-way ANOVA, LSD, Tamhane Test was used in normal hepatic tissue and latero-cancer tissue, and Mann-Whitney *U* in cancer tissue. in normal hepatic tissue

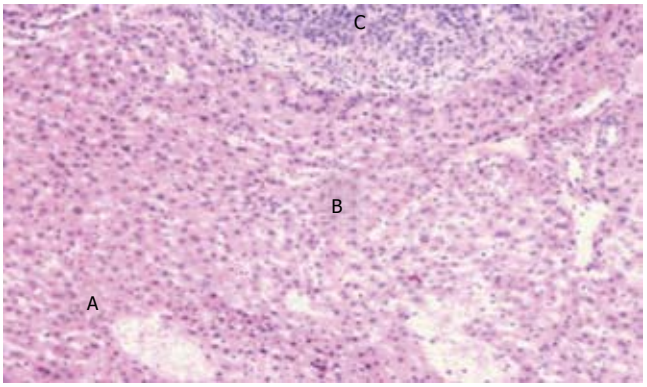


Figure 2 A: Hepatic tissue; B: Latero-cancer tissue; C: Cancer tissue, (HE, × 100).

(Test of Homogeneity of Variances, Levene Statistic = 2.18, $P = 0.104$; and One-way ANOVA Test, $F = 5.90$, $P = 0.002$), A > D, $P = 0.001$; B > D, $P = 0.031$; C > D, $P = 0.001$. It represented that the intensity of PTEN in Chinese crude drug group was higher than the Tegafur group. However, there was no significant statistic difference among Chinese crude drug group in difference concentrations ($P > 0.05$). In latero-cancer tissue, (Test of Homogeneity of Variances, Levene Statistic = 3.09, $P = 0.037$; and One-way ANOVA Test, $F = 15.99$, $P = 0.000$), A < B, $P = 0.170$; A > C, $P = 0.091$; B > C, $P = 0.017$; C > D, $P = 0.028$, it showed that expression intensity of PTEN under the intervention of Chinese crude drug was higher than that of Tegafur, the expression intensity of PTEN in medium dose group was the highest ($P = 0.05$). In cancer tissue, A < B, U = 30.5, $P = 0.016$; A > C, U = 44, $P = 0.104$; A > D, U = 24, $P = 0.005$; C > D, U = 18, $P = 0.002$; B < C, U = 9.5, $P = 0.000$; B > D, U = 4, $P = 0.000$. It represents that the intensity of PTEN in medium dose group was highest among the 3 kinds of tissue. In cancer tissue, A < B, $P = 0.014$; A > C, $P = 0.114$; A > D, $P = 0.005$; C > D, $P = 0.001$; B > D, $P = 0.000$, It also represented that the intensity of PTEN in medium dose group was highest ($P < 0.001$). However, there was no significant statistic difference between medium dose group and low group, low dose group and high dose group ($P > 0.05$) (Table 5).

Table 5 Expression intensity of PTEN of 3 kinds of tissue of the 4 groups (mean ± SD)

Group	*n*	Normal hepatic tissue	Latero-cancer tissue	Cancer tissue (median)
A	12	1.05 ± 0.34	1.42 ± 0.24	0.36
B	12	0.90 ± 0.31	1.75 ± 0.42	0.86
C	12	1.06 ± 0.18	1.27 ± 0.21	0.20
D	12	0.59 ± 0.48	0.64 ± 0.61	0.05

DISCUSSION

PTEN/MMAC1 (mutated in multiple advanced cancer 1) or TEP1 (TGF-regulated and epithelial cell-riched phosphatase1), located in human chromosome band 10q23[11], was recently identified together as tumor suppressor gene by three America research teams. As the first discovered dual-specific phosphatase, it may suppress tumor cell growth, regulate tumor cell invasion and metastasis through inhibiting many signal pathways of cell proliferation[12-15].

FT-207/tegafur, which belongs to the Second-generation fluoropyrimidine drugs, is metabolized to 5-fluorouracil through certain hepatic metabolizing enzymes and the cytochrome P450 (CYP450) system. Its anticancer mechanism is the same with that of 5-FU[16]. The advantage of Tegafur is highly fat-soluble, rapid gastrointestinal absorption, much longer half-life, as well as suitable for oral administration, its side effects is only one seventh that of 5-FU, while the efficacy index is twice higher. As the representative of the Second-generation 5-FU, Tegafur is used to treat several types tumors of alimentary canal as a routine chemotherapy drugs[17-19].

TCM includes primary hepatic carcinoma in the category of diseases such as: liver mass, abdominal mass, ZhengJia, tympanites, jaundice, *etc.* The etiological factors are concerned with yin-yang disbalance caused by the reception humid heat, long-term eating and drinking without temperance, being addicted to drink, internal injury caused by excess of seven emotions. When the vital qi gets deficient, evil factor easily invades the body and are stagnated in the liver cause the depression of liver-QI, activities of qi is stagnated and the blood circulation is blocked; Phlegm knobbing stagnant blood leads to the formation of abdominal mass, finally resulting in the formation of liver cancer[20,21]. The primary pathogenesis of liver cancer lies in the qi-stagnancy and blood stasis, the stagnation of humid heat, the discord of the spleen and the liver, as well as the weakness of vital qi[22]. At the early stage, this disease manifests itself in the unimpaired vital-qi, the type is most likely be sthenia syndrome or syndrome of blood stasis; at the middle stage, vital vital-qi is impaired, and asthenia and sthenia complicated with each other; at the advanced stage, the main syndrome is asthenia syndrome. Thus, the main treatment of TCM for liver cancer lies in strengthening body resistance and eliminating pathogen; the former is the mian way, the later is the assistant way[23,24]. Correlated investigation confirmed that the therapy of invigorating the spleen and regulating the qi could inhibit or delay the tumorous growth and metastasis, strengthen body's immunity, prolong the life span, and was more effective than the therapy of promoting blood flow and dissolving the stagnated blood and of[25,26], what's more, different therapeutic methods of TCM can in different degree regulate the transcription of some important oncogenes which play an important role in the process of occurrence and development of the liver cancer[27], thereby have some antitumous effect. Therefore, TCM becomes one of important combined therapies for cancer in China. In dealing with tumor, Chinese crude drugs have such advantages as following: guidance of wholism, strengthening the internal anticancer mechanism, mild toxicity, without pain, easily be accepted, it can relieve the symptoms, improve patient's function, lighten the toxicity and side effect meanwhile enhance effectiveness of the radiotherapy or chemotherapy, and accelerate recovery from operation, further more, can inhibit tumor growth, control or delay its recurrence, improve life quality, prolong

survival time. During to the traditionary chemotherapy wih the characteristic of being exist without tumor impacts the patient's exist quality, while people pay close attention to TCM wirh the characteristic of being exist with tumor.

FJD (Appl.: 200710026976.6) has been widely used to treat the hepatocellular carcinoma for years in our department. According to the special pathogenesis of middle and advanced hepatocellular carcinoma, the decoction can smooth the liver and strengthen the spleen, meanwhile can strengthen the body resistance, remove toxin and treat Biaol and Ben. So the decoction has the effect of strengthening the spleen and replenishing qi, diminishing stagnation by detoxification, relieving the depressed liver qi and regulating the blood. Previous research showed that this decoction had a certain anticancer effect on liver cancer cell lines *in vitro*[28] and in clinic[29]. In this study, we found that each TCM groups had distinct superiority in prolonging the live time and raising the survival rate of athymic mouse bearing cancer compared with the chemotherapy group. At the same time, middle and high concentration groups were more effective in prolonging the live time than that in low concentration group, which indicated TCM groups in prolonging the live time had dose-effect relationship. However, the TCM groups and the chemotherapy group has the similar effect in shortening tumor. Moreover, each group of the medicinal herbs were better than chemotherapy group chemotherapy group in inhibiting tumor diffusion and reducing tumor metastasis in liver. Compared with chemotherapy group, every TCM groups could reduce the number of tumor metastasis in liver, there was no difference between TCM groups and chemotherapy group in tumor metastasis rate in liver, which might relate with the insufficient the number of the sample. As mentioned previously, FJD could inhibit tumor metastasis and prolong the live time, the antitumous effect of FJD might through certain way to inhibiting tumor diffusion and metastasis other than shorten tumor, thus slowed down pathogenetic condition progressing, and prolonged the live time[30].

What on earth is the mechanism of the anticancer treatment by the Chinese herbs that strengthen the body resistance and remove toxic substances? Our research showed that the low, middle and high concentrations of the Chinese herbs could obviously increase expression of PTEN on the athymic mice bearing tumor respectively, compared with chemotherapy by ft207, which indicated the antitumous mechanism of medicinal herbs in this study. Interestingly, we found the expression of the PTEN in adjacent cancerous tissues of athymic mice bearing tumor was higher than that of the normal tissues, we inferred that this phenomenon might be a protection of the body itself to prevent cancer cell from further developing by high expression of PTEN in adjacent cancerous tissues under stressful condition. Therefore, the expression density of PTEN closely relates with the occurrence and development of liver cancer. Our study showed that FJD can provocate the expression of PTEN, and high expression of the PTEN in adjacent cancerous tissues seem to explain the reason why the intrahepatic metastasis rate in TCM groups is lower than that of chemotherapy group. The comparison among the TCM groups indicated that middle concentration achieved the best curative effect, low and high groups ranked secondly, this result illustrates that it is necessary for us to pay more attention to the dosage of medicinal herbs in clinic practice, because only the moderate dosage may acquire the best therapeutic effect. However, the best concentration of the FJD in treating HCC needs to be studied furtherly.

However, a control group of mice which were inoculated HCCs in the liver and were not given FZDJT and tegafur had been allocated. It's a pity that due to the too much cellular necrosis in pathological section we cannot get the result of immunohistochemistry. Next time we should do better.

In conclusion, our study showed that FJD can prolong the survival time and decrease tumor metastasis in livers of these experimental mice. Mechanisms of FJD healing HCC may partially be explained by enhancing the expression of PTEN in liver.

COMMENTS

Background

Traditional Chinese Medicine (TCM) which is useful to improve life quality, prolong survival time has been widely used as combined therapies in treating hepatocellular carcinoma (HCC). The primary pathogenesis of liver cancer lies in the qi-stagnancy and blood stasis, the stagnation of humid heat, the discord of the spleen and the liver, as well as the weakness of vital qi. Fuzheng Jiedu Decoction (FJD) (Appl.: 200710026976.6) has been widely used to treat the hepatocellular carcinoma for years. Previous research showed that this decoction had a certain antitumous effect on liver cancer cell lines *in vitro* and in clinic.

Research frontiers

TCM has been widely used as combined therapies in treating HCC in China. The novelty and innovation of the research consist in researching the mechanism of treatment of FJD to the hepatocellular carcinoma with mole-biological method. Mechanisms of FJD healing HCC may partially be explained by enhancing the expression of PTEN in liver.

Innovations and breakthroughs

TCM has been widely used as combined therapies in treating HCC, whether it can down-regulate the expression of PTEN in HCC is still unknown. The aim of our study is to explore the effect of FJD, complex prescription of Chinese crude drug in treating BALB/c athymic mice with HCC, as well as the expression of PTEN.

Applications

The mechanism of treatment of FJD to the hepatocellular carcinoma may partially be explained by enhancing the expression of PTEN tumor suppressor gene. We also found that different concentration of FJD had different effect in prolonging survival time and decreasing metastatic tumour. This study was indicated that FJD can be used as one of combined therapies in treating HCC and which was the best concentration of FJD.

Terminology

PTEN: Phosphatase and tensin homolog deleted on chromosome ten. FJD: Fuzheng Jiedu Decoction. FT207: Tegafur. TCM :Traditional Chinese Medicine.

Peer review

This is a good study in which the main objective is to explore the expression of PTEN in liver of athymic mice with HCC and the effect of FJD. PTEN was recently identified together as tumor suppressor gene. FJD can prolong the survival time and decrease Tumor metastasis in livers of these experimental mice. Mechanisms of FJD healing HCC may partially be explained by enhancing the expression of PTEN in liver.

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RAPID COMMUNICATION

A clinical trial of combined use of rosiglitazone and 5-aminosalicylate for ulcerative colitis

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Abstract

AIM: To investigate the therapeutic effects of the combined use of rosiglitazone and aminosalicylate on mild or moderately active ulcerative colitis (UC).

METHODS: According to the national guideline for diagnosis and treatment of inflammatory bowel disease (IBD) in China, patients with mild or moderately active UC in our hospital were selected from July to November, 2004. Patients with infectious colitis, amoebiasis, or cardiac, renal or hepatic failure and those who had received corticosteroid or immunosuppressant treatment within the last month were excluded. Following a quasi-randomization principle, patients were allocated alternatively into the treatment group (TG) with rosiglitazone 4 mg/d plus 5-ASA 2 g/d daily or the control group (CG) with 5-ASA 2 g/d alone, respectively, for 4 wk. Clinical changes were evaluated by Mayo scoring system and histological changes by Truelove-Richards' grading system at initial and final point of treatment.

RESULTS: Forty-two patients completed the trial, 21 each in TG and CG. The Mayo scores in TG at initial and final points were 5.87 (range: 4.29-7.43) and 1.86 (range: 1.03-2.69) and those in CG were 6.05 (range: 4.97-7.13) and 2.57 (range: 1.92-3.22) respectively. The decrements of Mayo scores were 4.01 in TG and 3.48 in CG, with a remission rate of 71.4% in TG and 57.1% in CG, respectively. Along with the improvement of disease activity index (DAI), the histological grade improvement was more significant in TG than in CG ($P < 0.05$).

CONCLUSION: Combined treatment with rosiglitazone and 5-ASA achieved better therapeutic effect than 5-ASA alone without any side effects. Rosiglitazone can alleviate colonic inflammation which hopefully becomes a novel agent for UC treatment.

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Key words: Peroxisome proliferators-activated receptor γ; Rosiglitazone; Ulcerative colitis

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INTRODUCTION

Ulcerative colitis (UC) is chronic intestinal inflammation with uneven relapsing course. Most patients need to take daily medications lifelong. The conventional therapies for UC include 5-aminosalicylate (5-ASA), glucocorticoid, and immunomodulator. 5-ASA and its derivatives were widely used to induce and maintain remission in patients with UC. But the general response rate was only 70%-80% and the relapse rate varied depending on the maintenance of the medications and other relapsing factors. It is necessary to search for special target of inflammatory cascades in UC so as to improve the therapeutic effects. With deeper understanding of its pathogenesis, more and more therapeutic targets have been found, and more and more novel clinical trials are directed to these targets with encouraging results. Peroxisome proliferators-activated receptor γ (PPAR-γ), highly expressed in the colon, is a subgroup of ligand-activated nuclear receptors responsible for the regulation of cellular events ranging from lipid homeostasis to cell differentiation and apoptosis. Recent studies showed its ligands can suppress the inflammatory response by inhibiting the activity of macrophages, cytokines production and NF-κB transcriptional activities. Thiazolidinediones (TZDs), such as rosiglitazon, are PPAR-γ synthetic ligands which have been used in type 2 diabetes mellitus for a long time. James *et al*[1] reported that refractory UC patients achieved clinical and endoscopic remissions using rosiglitazone, and concluded that it might represent a novel approach of UC therapy. However, it is an open-label trial with a small sample size and needs to be validated by expanded trials.

In this study we observed the therapeutic effects of combined use of rosiglitazone and 5-ASA in UC clinically and improvement pathologically.

MATERIALS AND METHODS

Design

Patients with mild or moderately active UC, who met with the criteria of suggested guidelines for the diagnosis and treatment of inflammatory bowel disease (IBD), were enrolled[2]. Patients with infectious colitis, acute or chronic cardiac, renal and hepatic failure and abnormal liver-associated chemistries were excluded. Patients who took glucocorticoid or immunomodulators, such as AZA, 6-MP, within the last month were also excluded. Included patients all provided informed consent. During the trial, the patients were not allowed to take traditional Chinese medicines (TCM), glucocorticoid and immunomodulator.

Assignment

The patients were assigned into treatment group (TG) or control group (CG), with even number allocated into TG and odd number into CG. Both groups took 5-ASA 2 g/d (500 mg granule, Beaufour Ipsen Pharmaceutical Co. Ltd), but rosiglitazone 4 mg/d (4 mg/tablet, GlaxoSmithKline Pharmaceutical Co. Ltd.) was added to the treatment group for 4 wk.

Follow-up

The patients were assessed from 0 to the 4th wk and interviewed by phone every week and returned for a visit at the 2nd wk, and received the colonoscopic examination and colonic mucosal sampling at initial and final points. The evaluation of disease activity was performed at 0 and the 4th wk according to the Mayo indices[3] (also called Sutherland index, Table 1).

Table 1 Mayo DAI indices

Stool frequency	
0	Normal number of stools for this patient
1	1 to 2 more stools than normal
2	3 to 4 more stools than normal
3	5 or more stools than normal
Rectal bleeding	
0	No blood seen
1	Streaks of blood with stool less than half the time
2	Obvious blood with stool most of the time
3	Blood alone passed
Findings of endoscope	
0	Normal or inactive disease
1	Mild disease (erythema, decreased vascular pattern, mild friability)
2	Moderate disease (marked erythema, absent vascular pattern, friability, erosions)
3	Severe disease (spontaneous bleeding, ulceration)
Physician's global assessment	
0	Normal
1	Mild disease
2	Moderate disease
3	Severe disease

Definition of outcome

Patients with a final DAI score of $\leqslant 2$ were defined to achieve clinical remission and patients with a final score $\geqslant 3$ but reduction in the DAI of $\geqslant 2$ were defined to achieve partial remission.

Safety analysis

All patients were included in the safety analysis. The adverse events and concomitant medication were carefully documented. Safety evaluations included vital signs, patients' symptoms, physical examination, hematology, serum biochemistry, fecal routine, and urinalysis. All the patients received these tests at the initial and the final points of the study.

Histopathology

At least 2 colon samples were taken from each patient at initial and final points of the trial through colonoscopy. Tissues were fixed in 10% buffered neutral formalin and embedded in paraffin. Four μm sections were stained with the hematoxylin and eosin (HE stain) for histological evaluation. Tissue slides were blindly assessed by experienced pathologists based on the Truelove-Richards histological grading system[4] as follows: 0 = no polymorphs, 1 = small number of polymorphs in the lamina propria with minimal infiltration of crypts, 2 = prominent polymorphs in the lamina propria with infiltration of > 50% of crypts, 3 = florid polymorph infiltrate with crypt abscesses, and 4 = florid acute inflammation with ulceration.

Immunohistochemistry

Labeled streptavidin-biotin (LsAB) methods were used for PPAR-γ and NF-κB p65 detection. Paraffin-embedded colonic tissue samples were dewaxed in xylene for 5 min twice, rehydrated in a series of ethanol (100%-70%) for 3 min each followed by rehydration in PBS for 30 min. After rehydration, the endogenous peroxidase was blocked with 0.3% hydrogen peroxide followed by antigen retrieval by autoclaving sections in citrate buffer pH 6.0 (10 mmol/L Na citrate). After antigen retrieval, the sections were stained using the above-mentioned kit according to manufacturer's recommendations, but with the following modifications. Sections were incubated with the primary antibody at 37℃ for 2 h. The following antibodies were used at the indicated dilutions: PPAR-γ (E-8: sc-7273, Santa Cruz, Santa Cruz, CA USA) 1:100 and NF-κB p65 (Boshide Bio Corp, Wuhan, China) 1:200. Each section had its own control using the secondary antibody only. Preimmune serum was initially used to ensure specificity of the signal with each of the antibodies. The lipoma resection sample was used as the positive control and the substitute monoclonal antibody of PPAR-γ with PBS as the blank control.

Statistical analysis

Statistical analysis was performed with the SPSS 10.0 software. The *t* test of the sample mean value of the designed group was used to examine the Mayo scores and positive cell numbers in the immunohistochemistry. The rank sum test was adopted for the histological grading. A P value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

We enrolled 42 patients with mild and moderately active UC into our trial and 21 patients each in TG and CG.

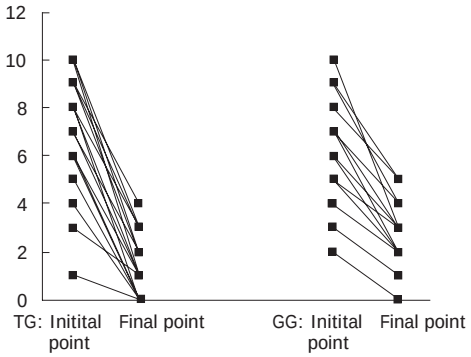


Figure 1 Mayo scores of TG and CG at initial and final points.

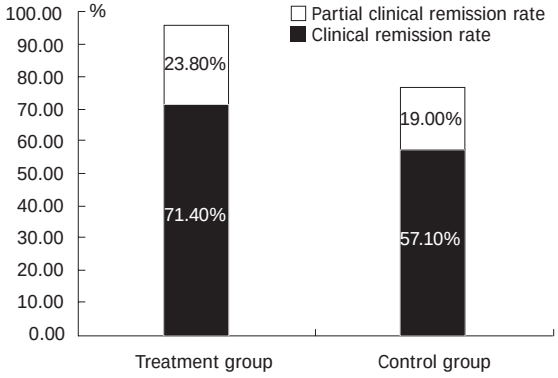


Figure 2 The clinical remission rate of TG *vs* CG.

The pre-study characteristics of patients are summarized in Table 2. The age ranged from 16 to 57 years (average, 38.8 ± 10.4 years) in TG and from 14 to 60 years (average, 37.1 ± 8.01 years) in CG, respectively. The extent of disease in TG was 8 proctitis (38.1%), 12 proctosigmoiditis (57.1%), and 1 left-side colitis (4.8%) and 9 (42.9%), 11 (52.4%), 1 (4.7%) in CG respectively. All patients received 5-ASA agents before enrolled into this study. The statistical analysis on the age, sex, location, type, and disease activity suggested similar baseline characteristics in the two groups ($P > 0.05$).

DAI scores at initial and final points

The Mayo score changes in each patient from 0 to the end of 4 wk were figured as below (Figure 1): the DAI in TG at initial and final points were 5.87 (range, 4.29-7.43) and 1.86 (range, 1.03-2.69), respectively, and those in CG were 6.05 (range, 4.97-7.13) and 2.57 (range, 1.92-3.22) respectively, (Table 3). There was a significant difference between the two groups and the therapeutic effect of TG was better than CG ($P < 0.05$).

According to the definition of outcome from the guideline, the clinical remission was achieved in 15 patients in TG (remission rate, 71.4%) and 12 patients in CG (remission rate, 57.1%), the partial remission was achieved in 5 patients (23.8%) and 4 patients (19.0%) in TG and CG respectively (Figure 2).

Histological grade at initial and final points

According to Truelove and Richards's histological grading system, the histological sections by H&E staining were graded in Table 4. The grade Ⅱ and Ⅲ of histology was found at the initial point (80.95%) and grade Ⅰ (57.14%) at the final points in TG, respectively, as compared with 33% grade Ⅰ at the final point in CG.

Immunohistochemistrical positive percentages at initial and final points

The positive percentage of PPAR-γ at initial and final points were 26.20%, 47.76% in TG and 24.90%, 39.24% in CG, respectively. The positive percentage of NF-κB were 58.09%, 21.19% and 58.00%, 27.00%, accordingly (Table 5). The relation between histological grade and immunohistochemistrical positive percentages was showed on Table 6.

Table 2 Clinical characteristics of ulcerative colitis

		TG (n = 21)	CG (n = 21)
Age (yr)		38.8 ± 10.4	37.1 ± 8.01
Sex	Male (%)	16 (76.2)	15 (71.4)
	Female (%)	5 (23.8)	6 (28.6)
Location	Rectum (%)	8 (38.1)	9 (42.9)
	Rectosigmoid (%)	12 (57.1)	11 (52.4)
	Left colon (%)	1 (4.8)	1 (4.7)
Type	Chronic relapse (%)	13 (61.9)	12 (57.1)
	Chronic persistence (%)	8 (38.1)	9 (42.9)
Severity	Mild (%)	5 (23.8)	6 (28.6)
	Moderate (%)	16 (76.2)	15 (71.4)

Table 3 Mayo scores of TG and CG at initial and final points

Mayo scores	Initial point	Final point
TG (n = 21)[a]	5.87 (4.29-7.43)	1.86 (1.03-2.69)
CG (n = 21)	6.05 (4.97-7.13)	2.57 (1.92-3.22)

[a]$P < 0.05$ *vs* CG.

Table 4 Histological grade of TG and CG

Grade	TG (n = 21)		CG (n = 21)	
	Initial point	Final point	Initial point	Final point
Ⅰ[a]	4	12	5	8
Ⅱ[c]	10	6	12	11
Ⅲ[e]	7	3	4	2
Ⅳ	0	0	0	0

[a]$P < 0.05$, TG *vs* CG; [c]$P < 0.05$, TG *vs* CG; [e]$P < 0.05$, TG *vs* CG.

Table 5 Positive percentages of PPAR-γ and NF-κB in TG and CG

	TG (n = 21)		CG (n = 21)	
	Initial point	Final point	Initial point	Final point
PPAR-γ[a] (%)	26.2	47.76	24.9	39.24
	(22.98-29.49)	(43.42-50.09)	(21.49-28.32)	(35.81-42.67)
NF-κB[c] (%)	58.09	21.19	58	27
	(54.51-61.68)	(19.13-23.25)	(53.21-61.02)	(21.08-32.92)

[a]$P < 0.05$, initial point to final point of TG *vs* CG; [c]$P < 0.05$, initial point to final point of TG *vs* CG.

Table 6 Histological grade and PPAR-γ and NF-κB expression

		Expression positivity (100%)			
		Ⅰ	Ⅱ	Ⅲ	Ⅳ
Initial points	*n*	4	10	7	0
	NF-κB	46.09-51.91	52.11-56.89	61.35-70.65	0
	PPAR-γ	31.57-39.93	24.54-29.06	16.30-20.55	0
Final points	*n*	12	6	3	0
	NF-κB	21.55-26.62	18.93-20.07	8.42-20.92	0
	PPAR-γ	37.20-44.97	49.82-56.51	55.50-69.84	0

Safety among treatment group and control group

During the observation period, no adverse events and abnormalities in the blood biochemical test were found in the patients. No jaundice or edema was found either.

DISCUSSION

As it is known, except for open-label trial by James *et al*[1], there are few clinical trials about rosiglitazone as adjunctive agent for UC conventional therapy. In this trail, treatment group showed a greater decrease in DAI and higher clinical remission rate than control group. Our results showed similar effects as those of James and his colleagues and the adjunctive effect of rosiglitazone on the UC therapy. However, we investigated the effects of rosiglitazone in mild and moderately active UC, but James did in refractory UC.

The PPAR-γ, highly expressed on adipo-tissue and colons, can regulate gene transcription of lipoprotein, cell differentiation, inflammation and immune response. PPAR-γ plays a key role not only in adipocyte differentiation, insulin sensitivity and nonalcoholic fatty liver[6-10], but also in innate and adaptive immunity[11-13]. Activation of PPAR-γ can promote macrophage desensitization, thus attenuating the oxidative burst[14]. Arnold and Konig[15] observed that PPAR γ ligands inhibited dose-dependently the release of TNF-α, GM-CSF, IL-1α, IL-6, IL-8 and CCL5 from RSV-infected A549 cells while diminishing the cellular amount of mRNA of IL-6, IL-8 and CCL5 and binding activity of the transcription factors NF-κB and AP-1, respectively. Belvisi *et al*[16] also outlined the anti-inflammatory effects of PPAR-γ ligands. Pan *et al*[17] revealed this anti-inflammation effect on human gallbladder epithelial cells as well. PPAR-γ ligands can prevent intestinal inflammation by blocking the activation of NF-κB, down-regulate the production of ICAM-1 and TNF-α in intestinal epitheliums, suppress expressions of TNF-α and IL-1β, *etc.*

Dubuquoy *et al*[5] found that expression of PPAR-γ in colon mucosa was decreased in active UC and was negatively related with UC severity. PPAR-γ was regarded as a new therapeutic target in IBD, especially in UC[18]. Christel *et al*[19] even found that the intestinal anti-inflammatory effect of 5-ASA depends on the PPAR-γ in chemically induced colitis in mice heterozygous at the PPAR-γ locus. These results strongly suggested a reasonable combination of these two agents for the therapeutic purpose on UC.

There are two kinds of PPAR-γ ligands, one is natural ligands such as prostaglandins J2, the other is synthetic ligands including TZDs. TZDs (e.g. rosiglitazone and pioglitazone) can reduce the productions of several pro-inflammatory cytokines and ameliorate the intestinal inflammation. Adachi M *et al*[20] reported that PPAR-γ in colonic epithelial cells plays an anti-inflammatory role and protects against experimental IBD. Marina Sánchez-Hidalgo *et al*[21] draws a similar conclusion. Sasaki *et al*[22] reported that troglitazone significantly reduced the TNF-α mediated induction of endothelial MAdCAM-1 in a dose-dependent manner; it also lowered the VCAM-1, ICAM-1 and E-selectin expression and significantly reduced α4β7-integrin dependent lymphocyte adhesion. Bassaganya *et al*[23] found that activation of PPAR-γ can inhibit the activation of NF-κB and ameliorate experimental colitis. NF-κB controls the transcription of a large cohort of genes, its dysregulated activation is linked to various biological disorders including inflammatory, and immune disorders[24]. In IBD, NF-κB plays an important role and is a target of various anti-inflammatory drugs. The ability of TZDs to ameliorate the experimental colitis is closely related to inhibition of NF-κB activation[19]. Another unpublished study of ours also investigated colonic mucosal expressions of PPAR-γ and NF-κB in the oxalzolone-induced experimental colitis, and found that the PPAR-γ ligand could increase the expression of PPAR-γ and inhibit the activation of NF-κB in colonic epithelium, ameliorating the colon inflammation in experimental colitis. Lawrence *et al*[25] showed that PPAR-γ ligands could provide anti-inflammatory protection by maintaining the cytokine balance and shifting transcriptional regulation of T cells away from Th1 and towards Th2 predominance in acute DSS colitis. Lytle *et al*[26] found that rosiglitazone could slow down the onset of spontaneous IBD in IL-10 (-/-) mice. Sánchez-Hidalgo *et al*[27] also reported that rosiglitazone for TNBS colitis can correct mucosal lesions, and significantly lower the ulceration index, myeloperoxidase (MPO), and the levels of TNF-α. Meanwhile, it increased prostaglandin (PG) E_2 production and returned PG D_2 to basal levels and reduced COX-2 and NF-κB proteins expression. Pedersen *et al*[28] reported that PPAR-γ mRNA and adipophilin expressions (a marker of PPAR activation) were markedly lower in colonic epithelium cells (CEC) from the patients with active UC and were clearly associated with the inhibition of PPAR-γ function and the stimulation with rosiglitazone fully restored PPAR-γ activation in CEC. Zhang YQ *et al*[29] reported that rosiglitazone enhances apoptosis of HT-29 cells by activating PPAR-γ. Li *et al*[30] reported troglitazone can not inhibit cell proliferation, and induce apoptosis in HepG2 cells, but down-regulate the expression of COX-2 mRNA and protein.

Our study showed the therapeutic effects of rosiglitazone on human UC, but it is required to demonstrate whether the therapeutic effects of rosiglitazone or other PPAR-γ ligands depend on PPAR-γ NF-κB signal pathway or on other inflammatory pathway, such as P38 MAPK, or toll-like receptor. A larger sample size clinical trail is also needed to confirm the effects of rosiglitazone on human UC.

Rosiglitazone has few adverse effects except for an ALT increase and exacerbation of cardiac failure induced

by the body fluid retention. In our study, we excluded the patients with hepatic, cardiac failure and closely observed related clinical and chemical changes. No adverse events were found in our study, including 4 patients who took rosiglitazone for 12 wk. Based on the above observation, we conclude that TZDs, ligands of PPAR-γ can be used in the UC as an adjunctive agent efficiently and safely.

COMMENTS

Background

The peroxisome proliferators activated receptor γ (PPAR-γ) is highly expressed in the colon and play a crucial role in intestinal inflammation. Regulation of colon inflammation by PPAR-γ has been well demonstrated in experimental colitis. Recently, rosiglitazone achieved quite well therapeutic effects in refractory ulcerative colitis (UC) patients in the study of James D. This result suggested that PPAR-γ is a hopefully novel target for UC treatment in the future.

Research frontiers

The hotspots in this field of studies include mechanism of PPAR-γ in intestinal inflammation and finding its high-affinity ligands.

Innovations and breakthroughs

An open-label trial of rosiglitazone for UC by James D *et al* was a first research of clinical application of thiazolidinediones (TZDs); however, their study merely focused on refractory UC. We mainly studied the patients with active UC and explored the mechanisms of PPAR-γ ligands.

Applications

PPAR-γ has multiple functions in the immune system and in some major inflammatory diseases such as atherosclerosis, inflammatory bowel disease and rheumatoid arthritis. If the ligands of PPAR-γ can be utilized for UC treatment, it will provide another therapeutic target and improve the effects of UC treatment.

Terminology

PPAR-γ is a member of nuclear receptors family, proved to be a key transcription factor of adipocyte differentiation, lipid and glucose homeostasis and an important target in type 2 diabetes and metabolic syndrome. Besides its role in metabolic tissues, it appears to be expressed in several other cells, including immune cells such as macrophages, dendritic cells, eosinophils, T cells and B cells. It was pointed to a role in the immune system and its new aspect was revealed and developed in parallel: its potential anti-inflammatory activity. DAI is an abbreviation of disease activity index and widely used to evaluate the conditions and therapeutic effect for UC. There are several DAI systems; however, Mayo system is widely accepted.

Peer review

The authors aim to investigate the therapeutic effects of rosiglitazone on mild or moderately active ulcerative colitis and explore the relation between PPAR-γ and intestinal inflammation and NF-κB. Their results revealed the ligand of PPAR-γ can alleviate the inflammation of UC and this effect may be related with depression of NF-κB by PPAR-γ activation. Rosiglitazone can alleviate colonic inflammation and hopefully become a novel agent for the treatment of UC. PPAR-γ may be another therapeutic target of UC in the future.

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RAPID COMMUNICATION

Comparison of ligase detection reaction and real-time PCR for detection of low abundant YMDD mutants in patients with chronic hepatitis B

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Abstract

AIM: To compare the ligase detection reaction (LDR) and real-time PCR for detection of low abundant YMDD mutants in patients with chronic hepatitis B infection.

METHODS: Mixtures of plasmids and serum samples from 52 chronic hepatitis B patients with low abundant lamivudine-resistant mutations were tested with LDR and real-time PCR. Time required and reagent cost for both assays were evaluated.

RESULTS: Real-time PCR detected 100, 50, 10, 1 and 0.1% of YIDD plasmid, whereas LDR detected 100, 50, 10, 1, 0.1, and 0.01% of YIDD plasmid, in mixtures with YMDD plasmid of 10^6 copies/mL. Among the 52 clinical serum samples, completely concordant results were obtained for all samples by both assays, and 39 YIDD, 9 YVDD, and 4 YIDD/YVDD were detected. Cost and time required for LDR and real-time PCR are 60/80 CNY (8/10.7 US dollars) and 4.5/2.5 h, respectively.

CONCLUSION: LDR and real-time PCR are both sensitive and inexpensive methods for monitoring low abundant YMDD mutants during lamivudine therapy in patients with chronic hepatitis B. LDR is more sensitive and less expensive, while real-time PCR is more rapid.

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Key words: YMDD mutants; Hepatitis B virus; Real-time PCR; Ligase detection reaction

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INTRODUCTION

Lamivudine is an effective antiviral agent for treatment of patients with chronic hepatitis B and advanced liver diseases[1]. However, long-term lamivudine monotherapy leads to emergence of lamivudine-resistant hepatitis B virus (HBV) mutants in some patients chronically infected with HBV[1,2]. The incidence is 16%-32% in the first year and increases to 38%, 57%, and 67% after 2, 3, and 4 years, respectively[3-6]. Resistance is associated with mutations in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif of the reverse transcriptase, which is part of the catalytic site of the HBV polymerase[7]. Viological breakthrough and alanine transaminase (ALT) elevation have been shown to occur 2-28 wk and 12-31 wk after the emergence of YMDD mutants, respectively[8-10]. Initially, YMDD mutants consist of minor populations. They gradually replace the wild-type virus, reaching a 100% lamivudine-resistant variant population, and this replacement occurs in parallel with the increase in HBV DNA load[10]. Sensitive methods for early detection of lamivudine-resistant mutants will be helpful for physicians to make clinical decisions in treatment of patients with HBV infection.

Several technologies have been developed for the detection of lamivudine-resistant mutants[11]. Although nucleotide sequencing of PCR products is widely used to detect lamivudine resistance, it is expensive and laborious, and can detect only mutant viruses representing at least 50% of the total virus population[8]. Inno-LiPA, pyrosequencing, real-time PCR, and ligase detection reaction (LDR) are able to detect low abundant YMDD mutants in the wild-type HBV[8,12-15]. However, only few studies have compared these methods.

We have previously compared real-time PCR and pyrosequencing for detection of YMDD mutants in patients with chronic hepatitis B[16]. In the present study, we compared LDR and real-time PCR for detection of low abundant YMDD mutants in mixed plasmids and clinical

samples from lamivudine treated patients with chronic hepatitis B.

MATERIALS AND METHODS

Plasmids and controls

Plasmids and controls were prepared as previously described[14,16]. In brief, three previously identified serum samples containing HBV with YMDD, YVDD and YIDD sequences were used as template and amplified by PCR. PCR products were cloned using pGEM-T systems (Promega, Madison, Wisconsin, USA), and clones were sequenced using ABI 3100 sequencer (Applied Biosystems, Foster, California, USA).

Patients and samples

Serum samples were collected from 196 patients with chronic HBV infection. All patients were treated with lamivudine for three months to three years and serum HBV-DNA levels were above 1.0×10^4 copies/mL by real-time PCR. Among these samples, 52 samples with YMDD mutants below 50% of total HBV population (determined by real-time PCR[14]) were selected for comparison of LDR and real-time PCR. All these 52 samples were found to contain only the YMDD variant by sequencing of PCR products, but found to contain YVDD or YIDD variants with real-time PCR or LDR.

Extraction and quantitation of HBV DNA

HBV DNA was extracted from serum samples using the HBV DNA extraction reagents (Fosun Diagnostics, Shanghai, China) according to the manufacturer's instructions. Serum HBV DNA levels were measured on ABI 7300 real-time PCR system (Applied Biosystems, Foster, California, USA) with quantitative real-time PCR reagents (Fosun Diagnostics, Shanghai, China), which was approved by the State Food and Drug Administration of China for *in vitro* diagnostic use.

Sequencing of PCR products

HBV DNA samples were prepared for sequencing by amplification with PCR as described by Allen *et al*[7]. HBV DNA extracted from serum samples was amplified by PCR. PCR products were purified with QIAquick PCR purification kits (Qiagen, Chatsworth, California, USA) and were eluted from the column with 80 μL of distilled deionized water. The DNA quality and concentration were determined by absorbance measurements at 260 and 280 nm and by gel electrophoresis on a 2.5% agarose gel. All sequencing reactions were performed on ABI 3130 DNA sequencer (Applied Biosystems, Foster, California, USA).

Ligase detection reaction

LDR was carried out as described by Xiao *et al*[15]. In brief, for one type of mutant (YIDD or YVDD), one common probe and two discriminating probes for mutant and wild-type YMDD were used in LDR, which was carried out in 20 μL of buffer, 1 pmol of each probe, and 5 μL of sample DNA. The reaction mixture was incubated at 94℃ for 2 min, before adding 15 U of thermostable Taq DNA ligase (New England Biolabs, USA), followed by 20 cycles of 30 s at 94℃ and 4 min at 65℃. Two PCR reactions were performed with the product of the LDR as template for 30 cycles at 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 45 s. The PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining.

Real-time PCR

Real-time PCR for detection of YMDD mutants was performed as previously described[14,16]. In brief, parallel reaction C, V and I were used to detect total HBV, YVDD and YIDD variants, respectively. The amplification was performed on ABI 7300 PCR system (Applied Biosystems, Foster, CA, USA) by incubating the reaction mixture (50 μL) at 50 degree for two minutes, followed by 5 min at 95 degree, 40 cycles of PCR amplification (94 degree for 20 s and 53 degree for 30 s). The reaction system was provided and optimized by Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The percentage of mutants in total virus was calculated by the following equations[14]:

(1) ΔCt = Ct of control - Ct of mutants

(2) Ratio of mutants to total virus = $2^{\otimes X\tau}$

Mixing experiments

Mixing experiments were carried out as previously described[16]. In brief, mutant plasmid containing YIDD sequence and wild-type plasmid were mixed at a final concentration of 10^6 copies/mL, and the percentage of the YIDD plasmid in the mixture was 100%, 50%, 10%, 1%, 0.1%, and 0.01%, respectively. The mixtures were analyzed by LDR and real-time PCR respectively. For real-time PCR, each mixture was analyzed five times, and the mean Ct value of the five runs was used to determine the ratio of mutant to total viruses. For LDR assay, each mixture was analyzed only once.

Time study

Two skilled technicians were selected to perform the assays. Time required for each assay was measured by direct observation during the procedures performed by the technicians, including the process of DNA extraction, amplification, detection, and analysis.

Cost analysis

Cost for each assay was estimated based on the prices of reagents in China. The cost of instruments and labors was not included.

RESULTS

Detection of mixed plasmids

Mixtures of plasmids contained YIDD and YMDD at different ratios were detected by LDR and real-time PCR, respectively. LDR detected YIDD, YIDD/YMDD in the mixtures containing 100%, 50%, 10%, 1%, 0.1% and 0.01% YIDD plasmid. Real-time PCR detected YIDD in the mixture containing 100% YIDD plasmid and YIDD/YMDD in the mixtures containing 50%, 10%, 1% and 0.1% YIDD plasmids, but detected only YMDD in the mixture

Table 1 Results of LDR and real-time PCR for detection of mixed plasmids containing YIDD and YMDD at a final concentration of 10^6 copies/mL

YIDD plasmid in the mixture	LDR	Real-time PCR
100% (10^6 copies/mL)	YIDD	YIDD
50% (5×10^5 copies/mL)	YIDD/YMDD	YIDD/YMDD
10% (10^5 copies/mL)	YIDD/YMDD	YIDD/YMDD
1% (10^4 copies/mL)	YIDD/YMDD	YIDD/YMDD
0.1% (1000 copies/mL)	YIDD/YMDD	YIDD/YMDD
0.01% (100 copies/mL)	YIDD/YMDD	YMDD

containing 0.01% YIDD plasmid (Table 1). It means that real-time PCR and LDR are able to detect 1000 and 100 copies/mL of mutant virus in the background of wild type viruses, respectively. The results of real-time PCR were consistent with our previous study with mixtures of YVDD and YMDD plasmids[16].

Comparison of LDR and real-time PCR for detection of clinical samples with low abundant YMDD mutants

We tested clinical serum samples from 52 lamivudine treated patients with chronic hepatitis B who had low abundant YMDD mutants. All the samples were detected as YMDD virus by sequencing the PCR products. The results obtained by LDR and real-time PCR were consistent (Table 2). Both methods detected 39 YIDD, 9 YVDD, and 4 YIDD/YVDD. The percentages of mutants in the virus population obtained by real-time PCR ranged from 4% to 40%. The percentage of the four YIDD/YVDD mixed mutants was 10%/20%, 30%/20%, 40%/10%, and 20%/30%, respectively.

Time required

In this study, we used 96-well PCR equipment and all the 52 samples were dealt with in a run. The total assay time for LDR and real-time PCR was 4.5 and 2.5 h, respectively.

Cost

The cost per test for each assay was calculated based on the prices of the reagents in China. Primers and probe were synthesized in TaKaRa Biotech (TaKaRa, Dalian, China). Real-time PCR mixtures were from Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The total reagent cost per test for LDR and real-time PCR was 60 and 80 CNY (8 and 10.7 US dollars), respectively. Although the cost of labors is similar in the same region, the cost of equipment used for LDR assay is much lower than that for PCR assay (5000 US dollars *vs* 60 000 US dollars).

DISCUSSION

Lamivudine has revolutionized the treatment of chronic hepatitis B. Lamivudine-resistant mutations in the YMDD motif of polymerase gene were detected in lamivudine treated and untreated patients with chronic hepatitis B[14,17-19]. Clinical breakthrough was observed 2 wk-7 mo after the emergence of YMDD mutations[8-10], causing considerable morbidity and mortality in those patients[20-24].

Table 2 Comparison of results obtained by LDR and real-time PCR for 52 clinical samples

Types	No. of samples (%)	
	LDR	Real-time PCR
YMDD	0 (0)	0 (0)
YIDD	39 (75)	39 (75)
YVDD	9 (17)	9 (17)
YIDD + YVDD	4 (8)	4 (8)

Lamivudine-resistant mutants are frequently preexisting variants in HBV-infected patients and are selected during lamivudine therapy. These resistant variants initially represent a minority of the quasispecies and gradually replace the wild-type YMDD variants[10]. Detection of low abundant lamivudine-resistant mutants in the background of wild-type HBV as early as possible is helpful for virological follow-up and diagnosis of resistance in the clinical setting.

To date, many assays have been used for detection of lamivudine-resistant mutants in patients with hepatitis B[11]. The differences in sensitivity, specificity, cost, and time required do exist in these methods. Real-time PCR is able to quantitatively detect a small portion of resistant mutants in HBV populations and LDR is a newly developed method for detection of low abundant mutants in the background of wild-type HBV. In the present study, we compared LDR and real-time PCR for detection of low abundant YMDD mutations in lamivudine treated patients. The results obtained by the two methods were completely concordant in all samples, and 39 YIDD, 9 YVDD, and 4 YIDD/YVDD variants were detected. The percentages of mutants in the virus population obtained by real-time PCR ranged from 4% to 40%. In the mixing experiment, LDR was able to detect as low as 0.01% (100 copies/mL) of YIDD plasmid, while real-time PCR only detected 0.1% (1000 copies/mL) of YIDD plasmid in the background of YMDD plasmid. This may be due to LDR employing two kinds of amplification cycles, 20 cycles of LDR and 30 cycles of PCR, in the testing process. These results suggest that LDR is more sensitive than real-time PCR. In addition, the cost of LDR is slightly lower than that of real-time PCR. However, real-time PCR is much more rapid and requires less manual work than LDR. Both methods are sensitive and inexpensive compared to other methods for detection of YMDD mutation[16]. Another advantage of the real-time PCR method is that it is able to calculate the ratio of mutants to total virus in samples[14]. This will be useful in the clinical studies on the dynamics of resistant mutants during lamivudine therapy.

Several antiviral agents, such as adefovir and entecavir, can provide effective therapies in patients with lamivudine-resistant HBV[25-27]. Pegylated interferon also induces sustained responses in a portion of lamivudine-resistant patients[28-30]. Monitoring low abundant YMDD mutation during lamivudine therapy by sensitive and inexpensive methods will be helpful for physicians to make better clinical decisions as early as possible in management of chronic hepatitis B.

In conclusion, both LDR and real-time PCR are sensitive and inexpensive methods for monitoring low abundant YMDD mutations during lamivudine therapy in patients with chronic hepatitis B. LDR is more sensitive and less expensive, while real-time PCR is more rapid.

COMMENTS

Background

Many assays have been used for detection of lamivudine-resistant mutants in patients with hepatitis B. The differences in sensitivity, specificity, and cost do exist in these methods. However, only a few studies have compared these methods.

Research frontier

Lamivudine-resistant variants initially represent a minority of the viruses and gradually replace the wild-type YMDD variants. Methods for detection of low abundant lamivudine-resistant mutants in the background of wild-type hepatitis B virus (HBV) as early as possible are helpful for diagnosis of resistance in the clinical setting.

Related publications

Shi *et al* and Xiao *et al* developed real-time PCR and LDR assays for detection of minority lamivudine-resistant mutants in patients with hepatitis B. However, they did not compare the clinical performance between the two methods.

Innovations and breakthroughs

This article compared LDR and real-time PCR for detection of low abundant YMDD mutations in lamivudine treated patients. Both assays are sensitive and inexpensive for monitoring low abundant YMDD mutations during lamivudine therapy in patients with chronic hepatitis B. LDR is more sensitive and less expensive, while real-time PCR is more rapid.

Applications

Both LDR and real-time PCR are suitable for early detection of lamivudine-resistant mutations in patients treated with lamivudine.

Terminology

Ligase detection reaction (LDR) detects nucleotide sequence by annealing and subsequent ligation of two oligonucleotides (probe and detector). Ligation of the probe and detector occurs only when the two bases on either side of the ligation site are complementary to the template. LDR is usually coupled with PCR for detection of low abundant point mutations.

Peer review

This study is of importance for the early detection of lamivudine-resistant HBV mutants in patients with chronic HBV infection. The experiments appear to be conducted very carefully and by an experienced team of investigators.

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CASE REPORT

Intermittent gastric outlet obstruction due to a gallstone migrated through a cholecysto-gastric fistula: A new variant of "Bouveret's syndrome"

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Abstract

Bouveret's syndrome, defined as gastric outlet obstruction due to a large gallstone, is still one of the most dramatic biliary gallstone complications. Although new radiological and endoscopic techniques have made pre-surgical diagnosis possible in most cases and the death rate has dropped dramatically, "one-stage surgery" (biliary surgery carried out at the same time as the removal of the gut obstruction) should be still considered as the gold standard for the treatment of gallstone ileus. In this case, partial gastric outlet obstruction resulted in an atypical and insidious clinical presentation that allowed us to perform the conventional one-stage laparatomic procedure that completely solved the problem, thus avoiding any further complications.

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Key words: Bouveret's Syndrome; Biliary gallstone; Gastric outlet obstruction; Biliary surgery

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INTRODUCTION

Gallstones are completely asymptomatic in the majority of patients (60%-80%)[1]. When they become symptomatic, biliary colic is usually the first manifestation to be encountered. Patients with mild symptoms have a higher risk of developing both common and less frequent complications[2].

Biliary fistula is a rare complication (3%-5%) that is frequently preceded by an episode of acute cholecystitis[3,4]. Biliary fistula is mostly encountered in the duodenum although it can occur anywhere in the gastrointestinal (GI) tract[5]. Finally, just 7%-10% of biliary fistulae cause gallstone ileus, an intestinal obstruction caused by a stone that has migrated through the fistula and stopped anywhere in the GI tract[6]. The terminal ileum and ileocecal valve are the most common locations[7], whereas gastric outlet obstruction from an impacted gallstone named "Bouveret's syndrome", is a very rare complication (1/10 000 cholelithisiasis)[8].

To our knowledge, this is the first case of incomplete gastric outlet obstruction manifested as a "waxing and waning" syndrome for a period of over 20 mo before diagnosis was defined.

CASE REPORT

A 79-year old woman was referred to our hospital when a recent oesophagogastroduodenoscopy (OGDS) revealed the presence of a foreign body with its maximum diameter > 4 cm, partially obstructing the gastric lumen, firmly attached to the antral greater curvature region where a fistula was partially explorative. The procedure was performed to investigate the patient's two-year old dyspepsia. This mild symptom alternated every two months with bouts of colic pain associated with self-

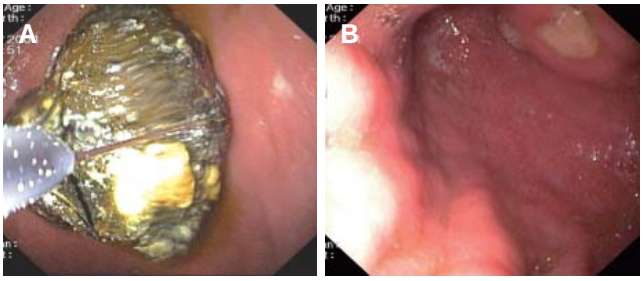


Figure 1 Oesophagogastroduodenoscopy confirming the presence of a foreign body looking like a biliary stone (**A**) and a 15 mm-wide ulcer (**B**).

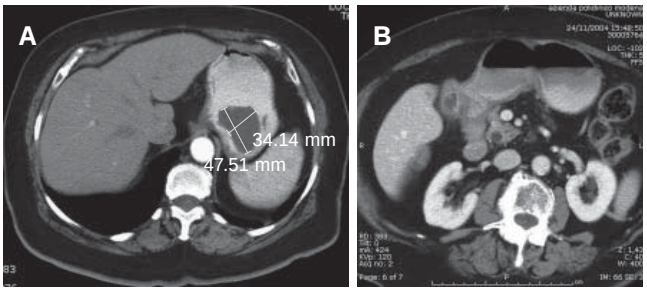


Figure 2 Computerised tomography revealing the density of foreign body typical of ectopic biliary stone (**A**) and the oedematous wall of the gastric antrum (**B**).

limited vomiting lasting 2 d.

The patient reported an earlier negative colonoscopy, performed due to the migration of gastric foreign body through a gastro-colic fistula. His symptoms occurred after two days of acute abdominal pain associated with vomiting due to acute gastroenteritis. An abdominal ultrasound scan (US) revealed cholelithiasis.

The patient was admitted to our ward in good general clinical conditions. Blood tests were completely normal apart from ferrittin, which was twice the normal range.

An abdominal computerised tomography scan (CT) and another OGDS were then performed. Endoscopic examination confirmed the presence of the foreign body, looking like a biliary stone (Figure 1A). Simple endoscopic lithotomy was not possible as the stone was too large, and an attempt at mechanical fragmentation of the stone was unsuccessful. The foreign body appeared to be firmly attached to the gastric wall, and a 15 mm wide ulcer was visible where the opening of the fistula has been previously described (Figure 1B). The CT revealed that the foreign body had a density typical of an ectopic biliary stone (Figure 2A) and the oedematous wall of the gastric antrum could not be separated from the thickened wall of the gallbladder fundus (Figure 2B).

Further air-fluid level was present in the gallbladder (Balthazar and Schester sign) and the intra- and extrahepatic bile ducts were slightly stretched. Lastly, a magnetic resonance cholangio-pancreatography (MRCP) was performed in the hope of observing the fistula. The procedure was negative (Figure 3). All of these data suggest that a multidisciplinary consultation was needed to decide the most appropriate management for this particular clinical setting.

We eventually opted to attempt "one-stage surgery".



Figure 3 Magnetic resonance cholangiopancreato-graphy showing no fistula.



Figure 4 One-stage surgery with dissection of omental-cholecystic adhesions.



Figure 5 A large ectopic stone extracted.

After dissection of many omental-cholecystic adhesions, retrograde cholecystectomy was performed successfully (Figure 4). Finally, a gastrostomy was performed on the anterior gastric wall and a large ectopic stone was extracted (Figure 5).

The post-surgical course was regular and during six-month follow-up the patients' general condition was excellent. The patient was completely asymptomatic with normal biliary tree on the US scan.

DISCUSSION

Like any other gallstone ileus, "Bouveret's syndrome" defined as gastric outlet obstruction caused by a large gallstone occluding the pyloro-duodenal region following a biliogastric or bilioduodenal fistula, primarily affects the geriatric population, with a medium age of 65-75 years and a female predominance (3-16:1)[9-11]. For these reasons, up to 80%-90% of patients have concomitant medical

illnesses (CIC, IRC, diabetes) and the management of such patients should be guided by their general clinical status[4].

The long period of approximately twenty months between the onset and diagnosis of the disease, can be attributed to the patients' carelessness, and the alternating gravity of symptoms combined with the patient's good general health can justify the decision to perform one-stage surgery.

In our case, the particular position of the gallstone led the patient to an insidious clinical setting that urged us to opt for "one-stage surgery" (namely, biliary surgery carried out at the same time as the removal of the intestinal obstruction), which should be still considered as the gold standard for the treatment of gallstone ileus[12]. In fact, it is the only procedure to solve the problem and avoid recurrences and other complications of gallstone disease[13].

In any case, over the past two decades, technological progress has probably made lithotomy or lithotripsy a more conservative approach to reducing patients' risks and has thus been suggested for older patients in poor medical conditions. In our patient, simple endoscopic lithotomy or lithotripsy was not indicated as the stone was excessively large (4 cm) and the inflammatory aspects of the gastric antrum required surgical treatment in order to close the fistula. An attempt at endoscopic mechanical fragmentation of the stone was unsuccessful.

The so-called "two stage surgery" consists of postponing biliary surgery (cholecystectomy and fistula repair) to a later stage with the presence of residual symptomatic stone in asymptomatic subjects[12,24].

Although no patients deny prompt relief from an intestinal obstruction[4], in the case of Bouveret's syndrome, an endoscopic approach represents a reliable alternative to gastro/duodenothomy[15,16]. Preoperative aetiological diagnosis is now possible in most patients and in this case, endoscopic lithotomy and lithotripsy (where possible) represent the first-line approach to treatment as the death rate associated with surgery is still 19%-24%[17,18].

Nevertheless, surgery remains the main procedure in particular situations such as stone impaction in the fistula[19], stone compression of the duodenal wall[20], GI haemorrhage[21] and improper stone manipulation[18,22-25].

In our case, the medical history and endoscopic findings suggested an unusual chronic situation to which the only possible solution was surgery. We, therefore, performed a complete radiological study to better define the anatomy of the lesion before the surgical procedure, including abdominal CT and MRCP of the biliary tree. The first combines the qualities of US and plain X-rays in defining Rigler's diagnostic triad (intestinal obstruction, pneumobilia and ectopic stone)[4,26]. It, therefore, represents the single best imaging technique for the diagnosis of gallstone ileus and Bouveret's syndrome[26]. It may also be helpful in assessing gallbladder wall thickness (signs of acute or chronic cholecystitis), content (air, residual gallstones) and biliary fistula[5,26].

MRCP has recently been proposed as a useful tool for differentiating between fluid and gallstones and also for observing the fistula when sufficient fluid is present[27].

In our case, all the investigations suggested a complicated clinical situation in which an incomplete pyloric occlusion developed following the migration of a large stone through a cholecystogastric fistula, probably due to a poorly-interpreted and treated episode of acute cholecystitis in a case of undiagnosed chronic cholecystitis.

In conclusion, the clinical picture and anatomy of the lesions suggest that a conventional one-stage laparatomic procedure can completely solve the problem, simultaneously avoiding recurrence and other gallstone disease complications.

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CASE REPORT

Case of clear-cell hepatocellular carcinoma that developed in the normal liver of a middle-aged woman

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Abstract

A 36-year-old woman was admitted to our department for close examination of a liver tumor that was found during a medical checkup. Abdominal US, CT and MRI showed a tumor in segment 7 (S7) of the liver. Although imaging suggested hepatocellular carcinoma, laboratory tests showed no abnormality in liver function, hepatitis virus markers were negative, and tumor markers including protein induced by vitamin K absence or antagonist Ⅱ (PIVKA-Ⅱ), α-fetoprotein (AFP), carbohydrate antigen 19-9 (CA19-9), and carcinoembryonic antigen (CEA) were all within normal ranges. Upon aspiration biopsy of the liver, the histopathological diagnosis was moderately differentiated hepatocellular carcinoma. Therefore, right hepatectomy was performed. Although a part of the tumor was necrotic, about 60% of the viable part showed a clear-cell variant. Consequently, it was diagnosed as clear-cell hepatocellular carcinoma. It was noted that the background liver tissue was normal. This case is worthy of reporting because development of clear-cell hepatocellular carcinoma in the normal liver of a middle-aged woman is rarely seen.

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Key words: Clear-cell hepatocellular carcinoma; Liver; Middle-aged

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INTRODUCTION

Hepatocellular carcinoma often develops against a background of chronic viral hepatitis including hepatitis B virus (HBV) and hepatitis C virus (HCV), autoimmune hepatic diseases including autoimmune hepatitis and primary biliary cirrhosis, alcoholic liver injury, and non-alcoholic steatohepatitis (NASH). We report a case of hepatic cancer in which no abnormality existed in liver function, hepatitis virus markers were negative, and the background liver was normal, and which was histopathologically clear-cell carcinoma, in which clear cells accounted for about 60% of the tissue. This patient presented with no background of carcinogenic risk factors, such as viral hepatitis, and showed rare histopathology.

CASE REPORT

A 36-year-old woman had a medical checkup on October 17, 2005. A tumor was found in S7 of the liver by abdominal US. She was referred and admitted to our department on November 28, 2005 for close examination and treatment. She had a history of alcohol consumption of 1 L beer per day for 15 years. Her family had no history of hepatic disease. On admission, her conjunctivas were not jaundiced, and heart and respiratory sounds were normal. The liver, spleen and tumor were not palpable.

Laboratory tests on admission showed no abnormality such as inflammation or abnormal liver function for peripheral blood and biochemical tests. Hepatitis B surface antigen (HBsAg), hepatitis B core antibody (HBcAb) and HCV antibody (HCVAb) were negative. All tumor markers tested showed normal values: specifically, 16 mAu/mL for PIVKA-Ⅱ (criterion, < 40), 6.2 ng/mL for AFP (criterion, ≤ 10.0), 20.2 U/mL for CA19-9 (criterion, ≤ 37), and 0.9 ng/mL for CEA (criterion, ≤ 5.0). Anti-nuclear and anti-mitochondrial antibodies were negative.

Abdominal US and CT showed a tumor of about 60 mm diameter in S7 of the liver. The lesion was visible on early phase imaging. However, there was also a part that was not visible in the image. It was apparent as a low-density area in the portal phase image (Figure 1A and B). Abdominal MRI showed that the T1-weighted image revealed a well-defined low-intensity area in S7, with a high-intensity part inside. The tumor was depicted as a

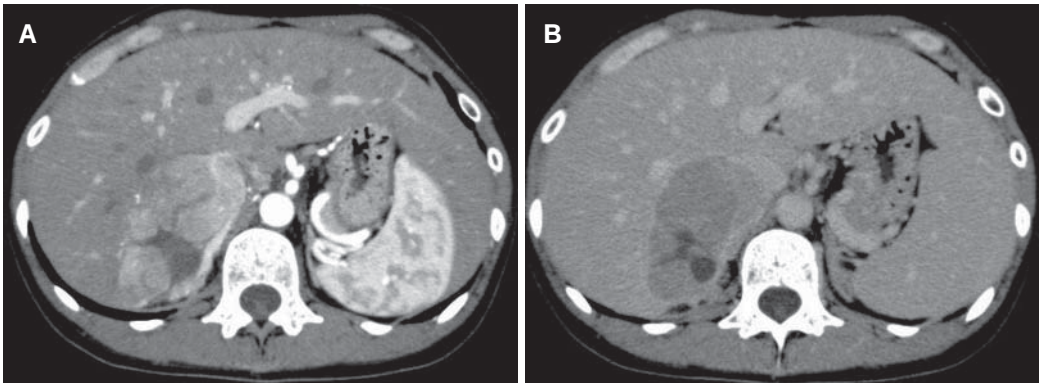


Figure 1 **A**: Dynamic enhanced CT showed a nodular lesion in the liver that was enhanced in the early image. However, the interior of the mass showed a low-density area; **B**: In the subsequent late phase, the lesion revealed washout of contrast enhancement.

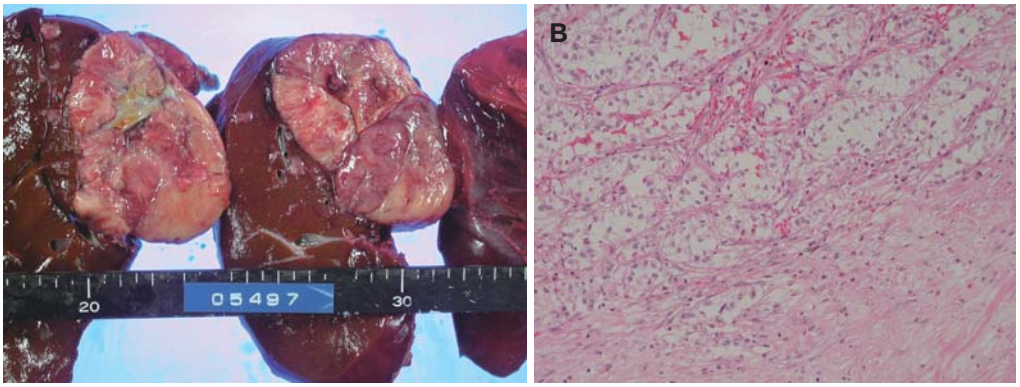


Figure 2 **A**: The tumor had a fibrous capsule. Its cut surface was whitish, and tumorous tissue had a necrotic part at the center; **B**: Histological examination showed sheet-like growth of clear, atypical cells (HE, × 40).

high-intensity area in the T2-weighted image. Angiography showed that increased tumor vascularity was observed from the right hepatic artery and the inferior phrenic artery to the tumor area. A slight tumor stain was visible in the tumor area in S7 of the liver. Although the imaging results suggested hepatocellular carcinoma, all hepatitis virus and tumor markers were negative, and non-cancerous tissues had no signs of chronic disease.

Consequently, aspiration biopsy of the liver was carried out for definitive diagnosis. Based on histopathology, the patient was diagnosed with moderately differentiated hepatocellular carcinoma, trabecular type, and right hepatectomy was performed on December 27, 2005. A 60-mm tumor was located in the posterior segment of the right hepatic lobe; the tumor was partly adherent to the caudal portion of the adrenal gland. The tumor had a fibrous capsule. Its cut surface was whitish, and tumorous tissue had a necrotic part at the center (Figure 2A). Histopathology showed the border between the tumor and the non-cancerous part formed a fibrous capsule. Approximately 60% of the tumor showed sheet-like growth of clear, atypical cells (Figure 2B). Other areas of the tumor were composed of moderately-differentiated HCC with a trabecular growth pattern. The tumor center was occupied by necrotic tissue.

DISCUSSION

Hepatocellular carcinoma often develops against a background of chronic liver disease. Specifically, 77.4% and 62.6% of cases in Japan are associated with chronic hepatitis and liver cirrhosis, respectively. Among cases with histopathological diagnosis, only 7.6% have normal liver as the background, as in this present case[1]. Furthermore, HBV- or HCV-antigen-positive liver cirrhosis accounts for about 80% of cases of liver cirrhosis; other causes include primary biliary cirrhosis, autoimmune hepatitis, alcoholic cirrhosis, NASH, and Budd-Chiari syndrome[2]. In the present case, although the patient had a history of alcohol consumption, there was no sign of alcoholic fibrosis in non-cancerous tissues. For that reason, it was presumed pathologically that the hepatocellular carcinoma developed from the normal liver. Liver cancer is considered to be comparatively rare in young persons, and patients < 35 years old account for 0.6% of the cases diagnosed with clinical hepatocellular carcinoma[1]. Moreover, according to data from 1994 to 1995 by the Follow-up Committee, Liver Cancer Study Group of Japan in 1998, 60 cases of liver cancer in young patients aged < 35 years old consisted of 12 HCVAb-positive (20%), 34 HBsAg-positive (57%), three positive cases each (5%) for HCVAb and HBsAg, and 11 virus-marker-negative cases (18%), which indicates that HBsAg is often positive among younger patients with liver cancer[3]. Although virus markers were negative in our case, reports also exist of occult HBV infection, in which serum HBV marker was negative, but HBV existed in the serum and liver tissue[4], and comparatively frequent incorporation of HBV in hepatocellular carcinoma developed in normal liver[5-7]. Therefore, involvement of viruses in this case is not implausible.

Clear-cell hepatocellular carcinoma is not frequent and has been reported to account for 7.5%-12.5% of all liver cancer cases[8,9]. The existence of clear cells, as well as fatty changes, is characteristic of well-differentiated hepatocellular carcinoma in the early stage; its frequency is presumed to

be decreased along with enlargement of the cancer[10]. In a large hepatocellular carcinoma, as that presented in this case, it has been reported that clear-cell hepatocellular carcinoma occurs at a frequency of 0.9%-8.8%[11]. The mechanism of development of clear cells is presumed to involve metabolic disorders and abnormalities of sugar metabolism for reasons including decreased portal blood flow and underdeveloped tumor arteries in the early stage of cancer[10,12]. Histopa-thologically, it is important to distinguish clear-cell hepatocellular carcinoma from liver metastases from other organs, especially renal cell carcinoma, and it can be distinguished by immunostaining[13]. As for the present case, the possibility of malignant tumors derived from other organs was clinically eliminated, and immunostaining of excised samples excluded liver metastasis from renal cancer. On the other hand, fibrolamellar hepatocellular carcinoma and epithelioid hemangioendothelioma are malignant liver tumors that are often seen in young persons without background liver diseases. However, the present case did not have the same histopathology as those diseases. Prognosis of clear-cell hepatocellular carcinoma has been reported to be better than[8,14], the same as, or worse than[9,12] that of common hepatocellular carcinoma. Further careful follow-up observations are needed in the future.

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CASE REPORT

Anaplastic carcinoma associated with a mucinous cystic neoplasm of the pancreas during pregnancy: Report of a case and a review of the literature

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Abstract

Oncogenesis of anaplastic carcinoma of the pancreas is a subject of controversy, because it shows sarcomatous nature with extremely poor prognosis. We herein report an unusual case of anaplastic carcinoma occurring with a recurrent mucinous cystic neoplasm in a 38-year-old female. A 10-cm retroperitoneal cystic mass was pointed out in the first pregnancy and a probable diagnosis of mucinous cystic neoplasm was made in October 2000. She refused surgery first and delivered her baby uneventfully. During her second pregnancy in 2002, however, she presented hematemesis and underwent urgent distal pancreatectomy, splenectomy and partial resection of the gastric wall where the tumor perforated. A diagnosis of borderline-type mucinous cystic neoplasm with ovarian-like stroma was made. Nine months later, CT visualized a recurrent cystic tumor near the pancreatic stump, which was subsequently resected. Pathology revealed that the tumor was composed of two different components of borderline-type mucinous cystic neoplasm and anaplastic carcinoma. The latter was intensely positive for vimentin, CD68, p53 and focally for cytokeratin, suggesting both sarcomatous and carcinomatous differentiation. She survived four years after the second surgery without tumor recurrence. Although the origin of anaplastic carcinoma has not been determined yet, it should be remembered that anaplastic carcinoma can occur in association with mucinous cystic neoplasm of more benign histology.

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Key words: Mucinous cystic neoplasm; Anaplastic carcinoma; Pancreatic tumor; Ovarian-like stroma; Pregnancy

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INTRODUCTION

Anaplastic carcinomas of the pancreas are rare, representing only 0.5%-7% of all non-endocrine malignances in this organ[1,2]. The origin of the tumor is considered pancreatic ductal cells because they are accompanied with areas of adenocarcinomas in most cases[2]. However, they also contain mesenchymal components such as spindle-shaped cells and osteoclastoid giant cells, reminiscent of having sarcomatous differentiation. The prognosis of pancreatic anaplastic carcinoma is extremely poor. The combination of immature adenocarcinoma and sarcoma components may portend an adverse clinical course.

Mucinous cystic neoplasms of the pancreas, on the contrary, have an indolent clinical course and a much better prognosis than anaplastic carcinoma if respectable[3]. Mucinous cystic neoplasms occur predominantly in women and often have an ovarian-like stroma that contains spindle cells commonly observed in the ovary. The association of anaplastic carcinoma with mucinous cystic neoplasm has rarely been reported to date.

We report an unusual case of pancreatic anaplastic carcinoma coexistent with recurrent mucinous cystic neoplasm in a 38-year-old female, who previously underwent distal pancreatectomy for a borderline-type mucinous cystic neoplasm during her second pregnancy.

CASE REPORT

A 38-year-old female patient with no previous medical history was introduced to our hospital in October 2000, for further examination of a retroperitoneal cystic tumor that was disclosed incidentally by routine ultrasonography (US) in the first trimester of her first pregnancy. She was completely asymptomatic. Magnetic resonance imaging (MRI) and US showed a large multiloculated cystic tumor with a thick capsule at the tail of the pancreas. It measured 10 cm in diameter and displaced the stomach

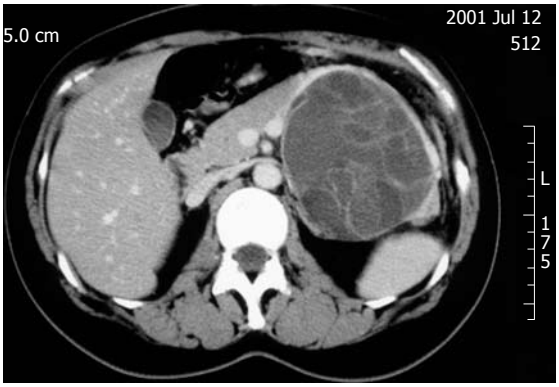


Figure 1 CT after the first delivery showing a 10 cm × 10 cm multi-loculated cystic tumor at the tail of the pancreas.

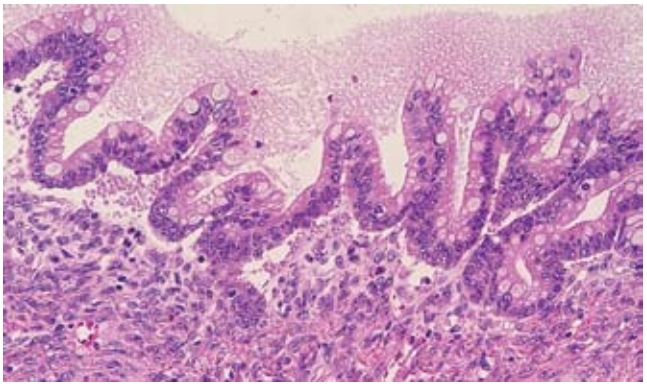


Figure 2 Consistent pathology of the primary tumor with a borderline-type mucinous cystic neoplasm (HE staining, original magnification, × 100).

anteriorly. A probable diagnosis of a mucinous cystic neoplasm was made. We recommended her to receive resection of this potentially malignant tumor soon, but she decided to postpone operation until delivery. She delivered a healthy baby in July 2001. Computed tomography (CT) showed a multiloculated cystic tumor (Figure 1), which seemed similar in size of that at the initial diagnosis. She hesitated to receive surgery and was lost to medical follow-up.

In September 2002, she presented repetitive vomiting and hematemesis, when she noticed her second pregnancy. In November, She presented hematemesis and tarry stool again. MRI and US showed marked enlargement of the cystic tumor. Because of progressive anemia, she underwent urgent operation in the second trimester. At laparotomy, a dense adhesion between the tumor and posterior wall of the stomach was noted, so that an en-block resection was accomplished by distal pancreatectomy, splenectomy and a partial resection of the stomach. The postoperative course was uneventful and she delivered her second baby in April 2003.

In August 2003, a routine medical checkup by US disclosed a 3 cm tumor near the pancreatic stump. The tumor showed both cystic and solid appearances with capsule enhancement. The second operation was performed under the diagnosis of local recurrence of the mucinous cystic neoplasm. At laparotomy, the tumor was located between the pancreatic stump, posterior wall of the stomach and the left adrenal, which was removed en-block by additional partial pancreatectomy, partial gastrectomy, and left adrenalectomy.

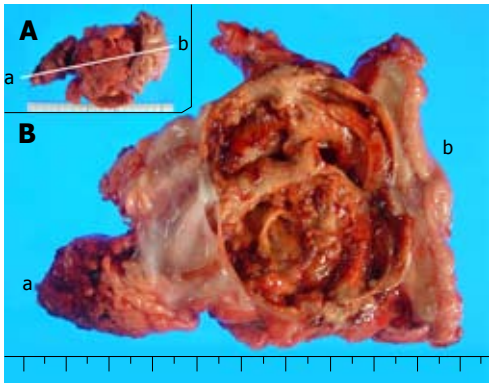


Figure 3 Cut surface of the recurrent tumor. **A:** Location of the cystic tumor between the pancreatic stump (a) and posterior wall of the stomach (b); **B:** Cut surface of the specimen revealing the tumor composed of two different components.

The patient was still alive and well four years after the resection. CT revealed no local and distant tumor recurrences at the latest follow-up.

Pathological findings

The tail of the pancreas resected at the first operation contained a multiloculated cystic tumor, measuring 14 cm × 14 cm, filled with about one liter of straw-yellow mucinous fluid. The cyst was uniformly lined with smooth inner surface with a thickened capsule of 5-10 mm width. At the cut surface of the maximal dimension, ten blocks were sampled for microscopic examination. The histological diagnosis was a mucinous cystic neoplasm, borderline type (Figure 2). The tumor had an ovarian-like stroma which was immunohistochemically positive for a progesterone receptor. None of the lymph nodes was involved, nor was the pancreatic cut margin. At the site of perforation to the stomach, no neoplastic change was identified in the gastric wall.

The recurrent tumor was entirely cystic and contained brown, dirty mucus. The tumor was located between the pancreatic stump and posterior wall of the stomach. Macroscopically, it was composed of two different parts. At the pancreatic side, the inner surface was white and smooth, resembling that of the previously resected primary tumor, while at the gastric side, it showed a tan-brown, irregular one. There was no transition area between the two parts (Figure 3). Pathology of the part near the pancreatic stump was a borderline-type mucinous cystic neoplasm. The part at the gastric side, on the other hand, contained an anaplastic carcinoma with osteoclastoid giant cells, atypical spindle-shaped cells and round cells, showing sarcomatous changes (Figure 4). The immunohistochemical the features of anaplastic carcinoma are summarized in Table 1. The recurrent tumor was also positive for progesterone receptor. All components of the anaplastic carcinoma were intensely positive for vimentin. Leukocyte common antigen and CD68 were positive in spindle-shaped cells and giant cells, while cytokeratin was positive in cytoplasm of the obviously epithelial component and

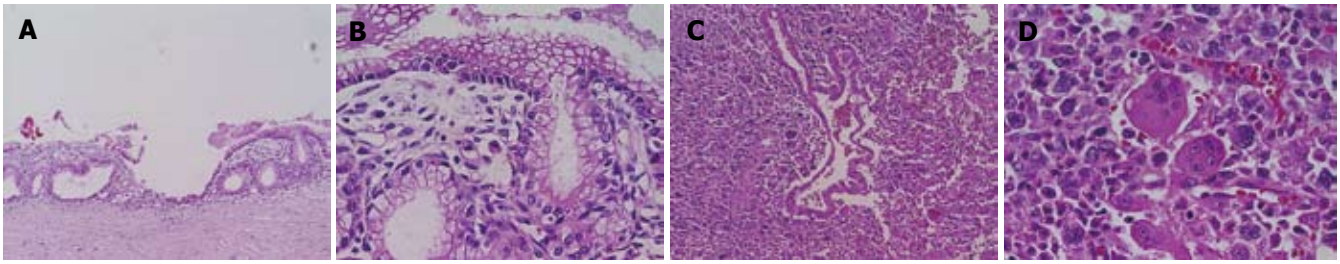


Figure 4 Pathology of the recurrent tumor at the pancreatic side (as pointed by a in Figure 3) revealing a borderline-type mucinous cystic neoplasm (HE, **A**: x 100, **B**: × 400) and at the gastric side (as pointed by b in Figure 3) an anaplastic carcinoma with giant cells, atypical spindle cells and round cells (**C**: × 100, **D**: × 400).

Table 1 Immunohistochemical features of each component in anaplastic carcinoma

	Adenocarcinoma	Spindle cell	Giant cell
Vimentin	+	+	+
Leukocyte common antigen	-	+ focal	+ focal
CD68	-	+	+
Cytokeratin	+	+ focal	-
P53	+	+	+

focally in some of the spindle-shaped cells. P53 was also positive throughout of anaplastic carcinoma.

DISCUSSION

Anaplastic carcinomas associated with mucinous cystic neoplasms are very uncommon. Only several cases have been reported to date (Table 2)[3-12]. Some underwent

Table 2 Reported cases of anaplastic carcinoma occuring in association with MCNs

No	Author (reference)	Case (age/sex)	Diagnosis	Anaplastic carcinoma							Outcome (survival time)
				Tumor location	Size (cm)	Spindle cell	Giant cell	Adeno-carcinoma	Invasion	lymphatic involvement	Distant metastasis	
1	Compagno J[3]	32/F	Anaplastic carcinoma derived from MCN	Body	ND	ND	ND	+	ND	ND	-	Dead (3 yr)
2	Gracia Rego JA[4]	45/F	Mucinous cystadenocarcinoma with pseudosarcomatous mural nodules	Body-tail	11	+	ND	+	Capsule	ND	-	Dead (16 mo)
3	Hartz PH[5]	56/F	Cystadenocarcinoma with anaplasia	Tail	6	+	+	ND	Capsule, peripan-creatic nerve	ND	Liver, peri-toneum	Dead (autopsy)
4	Lane RB[6]	25/F	Anaplastic carcinoma with MCN	Tail, liver, lymph node	15	+	-	+	ND	+	Liver	ND
5	Logan SE[7]	23/F	Pleomorhic adenocarcinoma originated from MCN	Body-tail	17	+	ND	+	Stomach	+	Liver	Dead (1 mo)
6	Marinho A[8]	70/F	Mucinous cystadenocarcinoma with a mural nodule of anaplastic carcinoma	Body-tail	4.5	ND	ND	+	Capsule	ND	-	ND
7	Sommers SC[9]	48/M	Cystadenocarcinoma with foci of pleomorphic carcinoma	ND	ND	ND	ND	+	+	ND	+	-	Dead (6 mo)
8	Tsujimura T[11]	43/F	Malignant histiocytoma with mucinous cystadenoma	Tail	16	+	+	ND	ND	ND	-	ND
9	[1]Wenig BM[12]	67/M	MCN with sarcoma stroma	Tail	19	+	ND	+	ND	ND	ND	Dead (15 mo)
10	[1]Wenig BM[12]	48/F	MCN with sarcoma stroma	Tail	ND	+	ND	+	ND	ND	ND	Alive (12 mo)
11	[1]Wenig BM[12]	65/F	MCN with sarcoma stroma	Tail, peritoneum	30	+	ND	+	Vascular	ND	Omentum, pleura	Dead (9 mo)
12	Hakamada K	39/F	Anaplastic carcinoma with recurrent MCN, borderline-type	Tail	5	+	+	+	-	-	-	Alive (4 yr)

ND: Not described; MCN: Mucinous cystic neoplasm. [1]Later re-analyzed genetically and reported by van den Berg W[11].

resection of the cystic tumor at the onset, while others were treated with cystenterostomy first. Because of co-existent adenocarcinomas in the areas of anaplastic cancer, most of the tumors were thought to be of epithelial origin. However, the pathogenesis of these neoplasms, particularly those with epithelial and mesenchymal components, remains a subject of controversy.

In our case, the entire anaplastic carcinoma was intensely immunohistochemical positive for vimentin, an intermediate fragment associated with mesenchymal cells. Areas of spindle-shaped cells and giant cells were also positive for leukocyte common antigen and CD60. These facts suggest the sarcomatous nature of the anaplastic carcinoma[12]. In addition to the positive vimentin, areas of the carcinoma were positive for cytokeratin. The co-expression of cytokeratin and vimentin in the cells with the same hisotological character seemed unusual, but it was reported that vimentin becomes positive when the tumor cells are in a proliferative state[13,14]. Immunohistochemical study alone cannot determine the origin of anaplastic carcinoma in the mucinous cystic neoplasms. Van den Berg *et al*[11] analyzed genetically three cases of anaplastic carcinoma related to mucinous cystic neoplasms, and disclosed that the sarcomatous and carcinomatous components are different phenotypically but genetically uniclone.

It was reported that malignant fibrous histiocytoma coexists with mucinous cystic adenoma[10], suggesting that sarcomas can occur from a relatively benign epithelial histology of mucinous cystic neoplasms. Indeed, the origin of sarcomas and carcinomas in mucinous cystic neoplasm is still unknown, but the theory that both sarcomas and carcinomas are originated from primitive mesenchymal stem cells with totipotential properties in the mucinous cystic neoplasm, might be another acceptable hypothesis for the histogenesis of anaplastic carcinoma.

Another interesting feature of this case is that the tumor experienced pregnancy twice. To the best of our knowledge, there are no reports on the relation of the progression of mucinous cystic neoplasm with pregnancy. Ovarian-like stroma exists exclusively in female patients with mucinous cystic neoplasms, which often are immunohistologically positive for progesterone and/or estrogen receptors. In our case, both the primary and recurrent tumors were positive for progesterone receptors. It is unknown whether physiological changes in blood concentration of these hormones promote the progression of the tumor. In our patient, the tumor size was stable during the first pregnancy, but rapidly enlarged in the second trimeseter of the second pregnancy. Increased mucious production might induce the enlargement of the tumor and subsequent rupture.

Our patient was still alive without any sign of recurrence four years after the second operation. However, close monitoring on tumor recurrence should be required because anaplastic carcinoma reportedly has a dismal prognosis.

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CASE REPORT

Exophytic inflammatory myofibroblastic tumor of the stomach in an adult woman: A rare cause of hemoperitoneum

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Abstract

Inflammatory myofibroblastic tumor (IMT) of the stomach in adults is extremely rare, with unpredictable prognosis. We present a 55-year-old woman with a gastric IMT. She experienced sudden abdominal pain 4 d previously. Physical examination showed mild abdominal tenderness in the hypogastrium, but no palpable abnormal abdominal mass. Abdominal CT showed a mass of approximately 8 cm in the gastrocolic ligament. On laparoscopic exploration, unexpected hemoperitoneum of approximately 1.5 L of blood was found, and an exophytic gastric mass of approximately 10 cm, appeared from the anterior wall of the gastric body along the greater curvature. Laparoscopy further showed that non-clotting blood in the abdominal cavity seemed to be from the gastric tumor. After conversion to open surgery for more precise evaluation of the cause of hemoperitoneum and the large friable tumor, gastric wedge resection, including the tumor, was conducted. The final diagnosis was consistent with IMT that originated from the gastric wall.

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Key words: Stomach disease; Stomach neoplasms; Hemoperitoneum; Myofibroma; Granuloma; Plasma cell; Stomach surgery

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Park SH, Kim JH, Min BW, Song TJ, Son GS, Kim SJ, Lee SW, Chung HH, Lee JH, Um JW. Exophytic inflammatory myofibroblastic tumor of the stomach in an adult woman: A rare cause of hemoperitoneum. *World J Gastroenterol* 2008; 14(1): 136-139

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INTRODUCTION

Inflammatory myofibroblastic tumor (IMT) is a rare neoplasm. The perspective of this distinctive disease has changed over time from a reactive, inflammatory process to a neoplasm of intermediate biologic potential[1,2]. Even though IMT has recently been known to span the entire age range and can arise from any site in the body[1], gastric IMT in an adult is, nevertheless, still very rare. We present a case of gastric IMT in an adult and a review of the literature.

CASE REPORT

A 55-year-old woman presented with acute-onset, sharp abdominal pain. The pain had developed 4 d prior to referral to our department from a local hospital for further evaluation of abdominal pain. On admission, her vital signs were normal. She denied other gastrointestinal symptoms and signs such as nausea, vomiting, abnormal bowel habits, melena or hematochezia, except for sharp abdominal pain. She also denied weight loss, fever, or other systemic symptoms. She had no medical or family history. Physical examination showed mild abdominal tenderness in the hypogastrium, but no palpable abnormal abdominal mass. Her laboratory findings, including tumor markers, were unremarkable, except for a normocytic, normochromic anemia: hemoglobin, 9.86 g/dL; hematocrit, 28.6%; mean corpuscular volume, 100.4 fL; and elevated erythrocyte sedimentation rate, 54 mm/h. The chest and abdominal X-ray films revealed no abnormal findings. Abdominal CT demonstrated a mass of approximately 8 cm in the gastrocolic ligament or gastric wall (Figure 1), and abdominal MRI showed a heterogeneous mass of approximately 8 cm in the gastrocolic ligament or gastric wall (Figure 2).

Endoscopic examination, including colonoscopy, showed no luminal or mucosal lesion and no remarkable

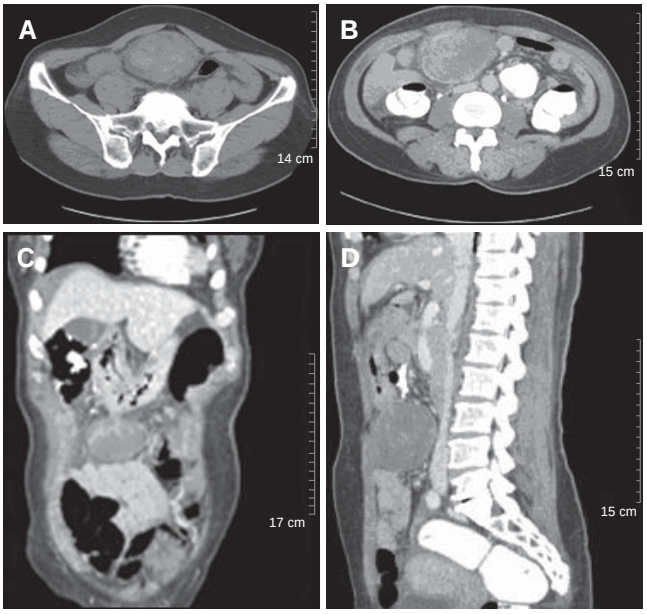


Figure 1 Abdominal CT revealed a large solid mass at the gastrocolic ligament or the gastric wall, which showed heterogeneous density on an non-enhanced image (**A**). The 8 cm mass showed internally enhanced vessels on the arterial phase of CT and delayed peripheral enhancement of the mass on the venous phase (**B**-**D**).

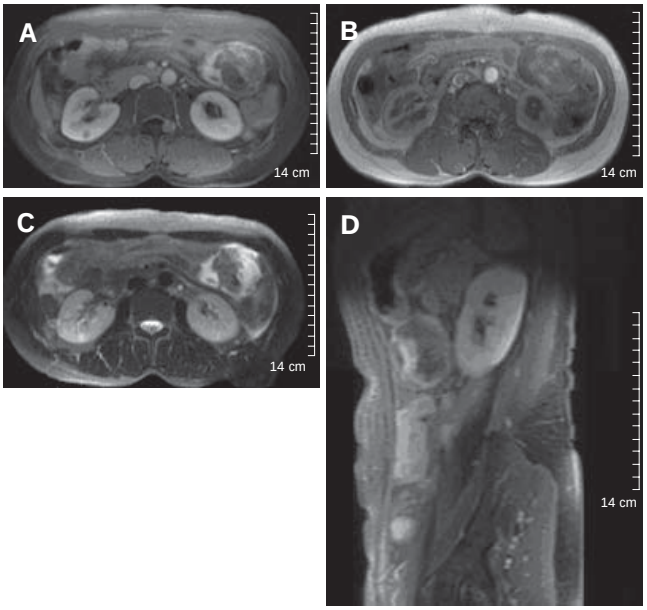


Figure 2 Contrast-enhanced MRI of the abdomen showed a mass of approximately 8 cm, seen at the left upper quadrant of the abdomen. The margin of the mass was lobulating, and it was attached to the greater curvature of the stomach. It contained a peripheral enhanced solid portion and a central non-enhancing portion (**A**). Signal intensity of the central non-enhancing portion was low on T1WI (**B**) and T2WI (**C** and **D**), which suggested internal hemorrhage within the tumor.

findings, except chronic atrophic gastritis with *Helicobacter* infection. Routine gynecological evaluation was unremarkable. After the work up for the intra-abdominal mass, her abdominal pain completely subsided at the time of operation.

The patient underwent laparoscopic exploration. Unexpected hemoperitoneum of approximately 1.5 L of non-clotting blood was found. An exophytic gastric mass from the anterior wall of the gastric body along the greater curvature appeared, similar in appearance to the spleen or a hematoma, approximately 10 cm in size (Figure 3). Laparoscopic diagnosis was massive intra-abdominal hemorrhage from the gastric tumor or ectopic spleen. Since further evaluation of the precise cause of hemoperitoneum was needed, and because it would have been difficult to remove the large friable tumor from the abdominal cavity, even after a safe laparoscopic resection we decided to convert to open surgery.

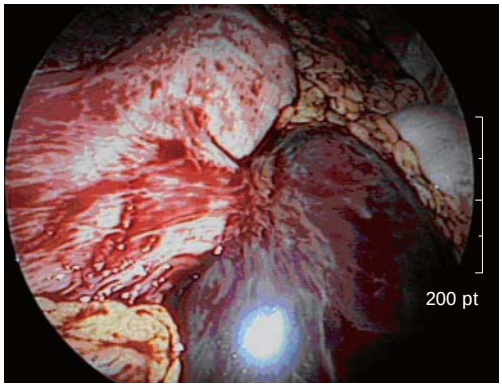


Figure 3 Laparoscopic view of the exophytic gastric mass with a large amount of intra-abdominal non-clotting hemorrhage.

After evacuation of blood, the abdominal cavity was thoroughly explored for any other source of bleeding, but none was found; the permeated or ruptured exophytic gastric mass was the cause of hemoperitoneum, because preoperative imaging studies, including gynecologic evaluation during admission, showed no evidence of intra-abdominal fluid, and also no other identified focus of bleeding upon surgical exploration. The tumor showed a dark reddish pedunculated mass with a stalk that originated from the gastric wall, but there was no blood vessel directly into the tumor. During dissection, we could not find any tear or rupture on the exophytic tumor. Gastric wedge resection, including the tumor and greater omentum, was conducted.

Grossly, the external surface of a well-encapsulated lump of soft solid tumor was smooth and glistening, but there was no gastric mucosal lesion (Figure 4A). The tumor measured 8.5 cm × 7.1 cm × 3.6 cm and weighed 88.1 g, and its stalk measured 2.0 cm × 1.2 cm × 1.9 cm. On serial sectioning, the cut surface was characterized by several amorphous fragments of parenchymal tissue, which were separated by the cystic spaces (Figure 4B). Histologically, the tumor was composed of round and spindle-shaped myofibroblastic cells, diffusely scattered inflammatory cells, and many vascular structures (Figure 5A). The mitotic count was 1/10 high power fields (HPF). The tumor cells showed positive immunoreactivity for vimentin (Figure 5B), while being negative for c-kit, CD34, desmin, smooth muscle actin (SMA), S-100, anaplastic lymphoma kinase (ALK), β-catenin, and CD31. Ki-67 labeling index was approximately 10%. Lymph nodes found along the gastroepiploic vessels in the omentum were all negative for tumor. The final pathologic diagnosis was consistent with IMT that originated from the gastric wall.

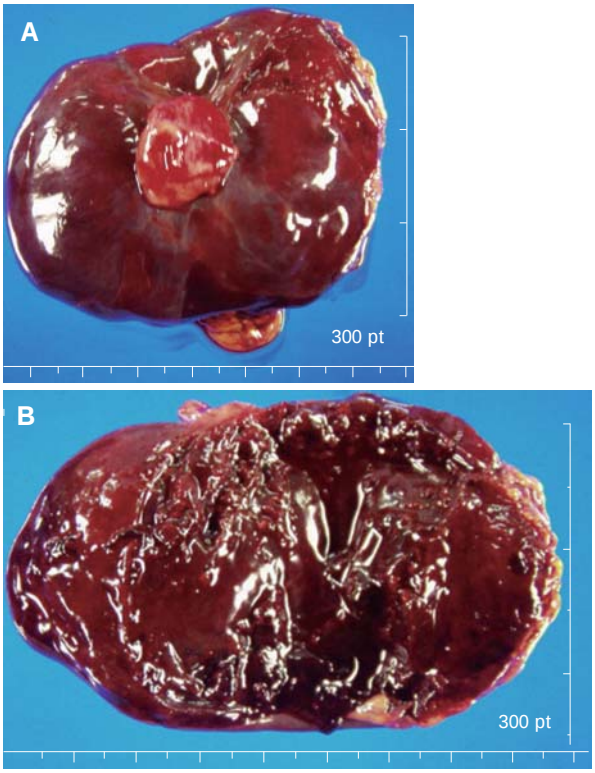


Figure 4 The external surface of a well-encapsulated lump of soft solid tumor, weighing 88.1 g, was smooth and glistening, but showed no gastric mucosal lesion (**A**). Cross-sectional surface of the tumor was characterized by several amorphous fragments of parenchymal tissue, which were separated by the cystic spaces (**B**).

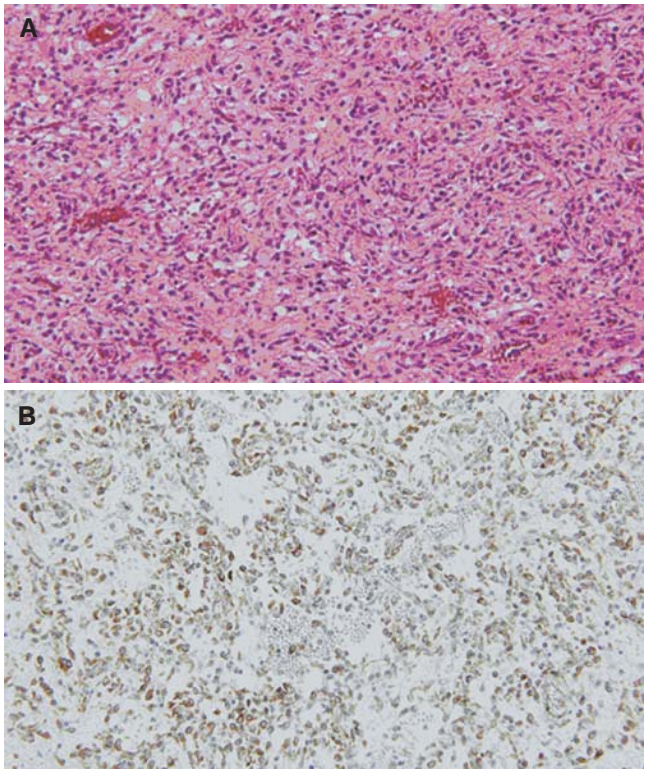


Figure 5 **A**: The tumor was composed of round and spindle-shaped myofibroblastic cells. Diffusely scattered inflammatory cells and many vascular structures are seen (HE, × 400); **B**: The tumor cells showed positive immunoreactivity for vimentin (× 400).

The patient had an uneventful postoperative course and has been followed up, including positron emission tomography, for any recurrence.

DISCUSSION

IMT is a rare, distinctive disease. Various terms such as inflammatory pseudotumor, plasma cell granuloma, inflammatory myofibroblastoma, and inflammatory myofibrohistioblastic proliferation have previously been used to describe the disease[3], which indicates that the exact nature of IMT is not yet fully understood. It has been debated whether IMT is a tumor or inflammation, and also whether it is benign or malignant[1]. However, recent studies on cytogenetic abnormalities, such as rearrangements of the ALK gene on chromosome 2p23[4,5], clonal chromosome abnormalities[6-8], and DNA aneuploidy[9], and the role of oncogenic viruses[10,11] in the pathogenesis of IMT suggest that it is a true neoplasm. According to the current classification of the World Health Organization[12], IMT is a neoplasm with a tendency for local recurrence and a very low rate of metastasis, and is histopathologically composed of myofibroblastic spindle cells, with inflammatory cell infiltrate of plasma cells, lymphocytes and eosinophils.

It was once accepted that IMT is primarily a disease of children and young adults and commonly occurs in the lungs[13,14]. However, a recent study by Coffin *et al* has shown that IMT may span the entire age range and can occur in any site of the body[1]. Nevertheless, gastric IMT in adults is still a very rare disease. Only four case reports of gastric IMT in adults exist in the English literature: Kim *et al* have reported a gastric IMT with peritoneal dissemination in a young adult[15]; Al-Taie *et al* have reported a rapidly growing inflammatory tumor after triple therapy for benign gastric ulcer[16]; Leon *et al* have experienced an IMT of the gastric remnant in a 50-year-old woman with a prior gastrectomy[3]; and Kojimahara *et al* have described a large, poorly demarcated, elevated IMT with infiltrative proliferation of spindle cells over the full thickness of the gastric wall in a 19-year-old woman[17].

This is believed to be the first case report of an exophytic gastric IMT that spontaneously bled into the peritoneal cavity and developed into hemoperitoneum. The present case can be compared with other intra-abdominal IMTs that present with an abdominal mass and related compressive symptoms, such as abdominal pain and vomiting[3,15-18].

Most IMTs require surgery to obtain definite diagnosis and cure. Complete resection is the preferred option, because incomplete excision has been shown to be a risk factor for recurrence[13]. As evident in this case, microrupture of the solid tumor might be another risk factor for early recurrence.

The main difficulty in the management of IMT lies in the unpredictable postoperative course. There are no definitive clinical, histopathological, or genetic features to predict recurrence or metastasis. Recently, reactivity of ALK has been reported to be a favorable prognostic indicator[1]. Differentiation between aggressive and non-

aggressive forms of IMT remains to be further clarified.

We reported a case of gastric IMT that spontaneously bled into the peritoneal cavity during admission. The patient is currently undergoing careful follow-up, because it is not clear whether gastric IMT is benign.

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CASE REPORT

Hyperinsulinemic hypoglycemia due to diffuse nesidioblastosis in adults: A case report

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Abstract

Persistent hyperinsulinemic hypoglycemia is caused most commonly by an insulinoma in adults or by nesidioblastosis in neonates. In adults, nesidioblastosis is a rare disorder characterized by diffuse or disseminated proliferation of islet cells. We recently encountered a case of nesidioblastosis in an adult. A 71-year-old man was admitted due to intermittent general weakness, abdominal pain, and mild dyspnea. The patient underwent a subtotal gastrectomy for a gastric adenocarcinoma two years ago. After 5 d of admission, the patient showed symptoms of cold sweating, chilling, and hypotension 30 min after eating. Thereafter, he frequently showed similar symptoms accounting for hypoglycemia regardless of food consumption. Laboratory findings revealed a low fasting blood glucose level (25 mg/dL), and a high insulin level (47 μIU/mL). Selective intra-arterial calcium stimulation with hepatic venous sampling (ASVS) was performed to localize a mass and revealed an increased insulin level about four-fold that of the normal fasting level at 60 s in the splenic artery, which suggested the presence of an insulinoma in the tail of pancreas. A distal pancreatectomy was performed. Neither intraoperative exploration nor a frozen biopsy specimen detected any mass-forming lesion. On the histological examination, many of the islets were enlarged and irregularly shaped in all specimens, the arrangement of which was a lobulated islet pattern. Cytologically, a considerable subpopulation of endocrine cells showed enlarged and hyperchromatic nuclei. By immunohistochemistry, the cells were identified as β-cells. These clinical, radiological, microscopic and immuno-histochemical findings are consistent with diffuse nesidioblastosis in adults.

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Key words: Hyperinsulinemic hypoglycemia; Nesidioblastosis; Adult

INTRODUCTION

Nesidioblastosis is a term originally conceived by Laidlaw[1] who described the neoformation of the islets of Langerhans from the pancreatic ductal epithelium. This disease is a rare disorder of infants characterized by persistent hypoglycemia as a result of hypersecretion of insulin from β-cell hyperplasia of the pancreas[2]. First described in neonates, it is widely recognized as the primary cause of persistent hyperinsulinemic hypoglycemia in infants[3]. However, in adults, hyperinsulinemic hypoglycemia is caused mostly by an insulinoma, and onset nesidioblastosis in adults represents 0.5%-5% of cases of organic hyperinsulinemia[4,5]. Since the first reported series of onset nesidioblastosis in adults by Harness *et al* in 1981[6], limited cases have been reported to date[7-9].

We report herein a very rare case of hyperinsulinemic hypoglycemia of an elderly man, which was negative in a localizing test for mass and positive in a selective arterial calcium infusion (SACI) test. He was found to have nesidioblastosis during a partial pancreatectomy.

CASE REPORT

A 71-year-old man was admitted to our hospital due to intermittent general weakness, abdominal pain, and mild dyspnea. The patient underwent a subtotal gastrectomy for a gastric adenocarcinoma 2 years ago. He had no history of diabetes mellitus or hypoglycemia. The patient had discomfort of the abdomen accompanying general weakness several months ago. After 5 d of admission, the patient showed abrupt symptoms of cold sweating, chilling, and hypotension 30 min after eating. These symptoms were relieved after intravascular administration of 50% glucose. Thereafter, the patient frequently showed similar symptoms accounting for hypoglycemia regardless of food consumption.

Laboratory findings determined when the symptoms were present revealed a low fasting blood glucose level (25-48 mg/dL), and a high insulin level (38-47 μIU/mL). A

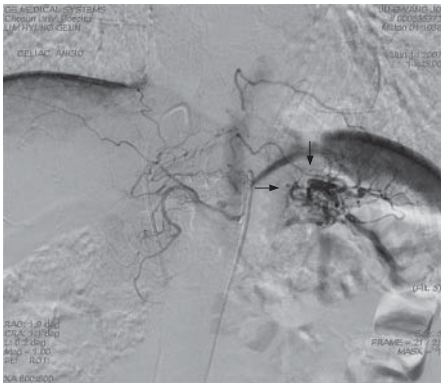


Figure 1 Celiac angiography showing a hypervascular mass-like lesion in the pancreatic tail area (arrows).

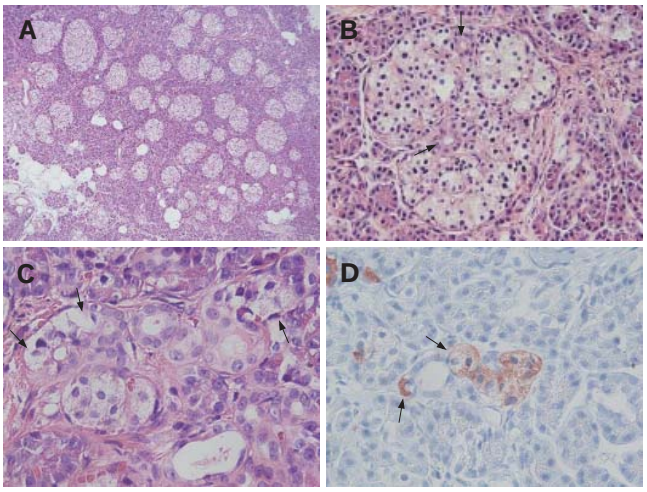


Figure 2 Irregularly sized-dysplastic islets scattering randomly throughout the pancreas (**A**), islets in intimate association with ducts forming a so-called ductulo-insular complex (arrows indicate ductules within the islet) (**B**), islet cells (arrows) budding off the duct epithelium (**C**), insulin-positive islet cells (arrows) budding off the duct epithelium (immunohistochemical stain for insulin) (**D**).

72-h fasting glucose study failed because of the occurrence of hypoglycemic shock 4 h after commencement of the test.

Endocrine examinations to exclude other causes of hypoglycemia, such as hypopituitarism and adrenal insufficiency, were within the normal range. The above-described symptoms and the results of serological tests were consistent with those of insulin-producing lesions including an insulinoma. However, imaging studies including computed tomography and a sonogram of the abdomen failed to detect a mass except for a highly vascularized area in pancreatic tail (Figure 1). To exclude any possible occult insulinoma, selective intra-arterial calcium stimulation with hepatic venous sampling (ASVS) was performed. After calcium gluconate (0.05 mg/kg body weight) was injected into the splenic, hepatic, gastroduodenal, and superior mesenteric arteries, blood samples were collected from the right hepatic vein every 30 s for 120 s. A selective arterial calcium injection test (SACI) to the left splenic artery increased the insulin level of about 4-fold over the pre-stimulated level. In contrast to the left splenic artery, no significant increment was induced by the SACI to the hepatic, gastroduodenal, and

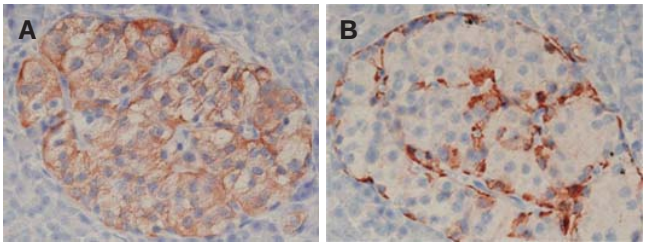


Figure 3 An immunohistochemical study showing increased insulin producing β-cells (**A**), glucagon producing α-cells (**B**).

superior mesenteric arteries, suggesting the presence of an insulinoma in the tail of pancreas. Under the assumptive diagnosis of an insulinoma of pancreatic tail based on the ASVS test, a distal pancreatectomy was performed. Neither intraoperative exploration nor a frozen biopsy specimen detected any mass-forming lesion. Grossly, the resected pancreas appeared normal, and there was no mass lesion in serial section specimens.

In a histopathological study, a number of dysplastic islets were randomly scattered throughout the pancreatic parenchyma, and their contour and size were markedly variable as compared to the normal pancreatic parenchyma. Ductuloinsular complexes and insulin-positive cells budding off the duct epithelium were also observed (Figure 2). Focally, the distribution of islets was densely crowded. In the majority of islets, multiple β-cells with enlarged and hyperchromatic nuclei and abundant clear cytoplasm were identified (Figures 2 and 3). Immunohistochemically, the number of insulin-secreting β-cells was increased, and the number of glucagon-secreting α-cells was decreased (Figure 3). These clinical, radiological, histopathological, and immunohistochemical findings were consistent with those of nesidioblastosis.

In the post-operative course, the glucose and insulin levels in the patient were well controlled and uneventful for two weeks after surgery. However, beyond that time, the patient repeatedly showed hyperinsulinemic hypoglycemia with no response to medication.

DISCUSSION

Nesidioblastosis is the name given to the presence of islets in intimate association with ducts, formation of so-called ductulo-insular complexes[10,11]. In adults, an insulinoma accounts for most cases of hyperinsulinemic hypoglycemia[4,12]. Nesidioblastosis has mainly been described in neonates[3]. Since Harness *et al*[6] first described nesidioblastosis in adults, it has only been reported in association with other diseases, such as Zollinger-Ellison syndrome, multiple endocrine adenomatosis, β-cell adenomatosis, Lindau's disease, cystic fibrosis, insulinomas, pancreatic transplantation, orbital lymphoma with hypopituitarism and adrenal insufficiency, familial adenomatous polyposis, hypergastrinemia, and pancreatic polypeptidemia[7].

The morphological criteria[13,14] for establishing its diagnosis are the presence of differently-sized islets often with somewhat irregular outline, and irregularly sized and poorly defined endocrine cell clusters scattered in the acinar parenchyma and often intimately connected with

small or large ducts (ductuloinsular complexes). Another feature is a distinct islet cell hypertrophy with nuclear enlargement, often resulting in the presence of giant and bizarre nuclei. As seen by immunohistochemistry and electron microscopy, these cells are found to be insulin-producing cells. The histopathological findings in the present case were compatible with these criteria. Nesidioblastosis is classified into focal and diffuse types characterized by different clinical outcomes[15]. Focal nesidioblastosis exhibits nodular hyperplasia of islet-like cell clusters, including ductuloinsular complexes and hypertrophied insulin cells with giant nuclei. In contrast, diffuse nesidioblastosis involves the entire pancreas with irregularly sized islets[10].

Sporadic hyperinsulinemic hypoglycemia is the main clinical feature of nesidioblastosis. The present patient had frequently hypoglycemic episodes mostly in the afternoon and night, independent of food consumption. It should be noted that a gastrectomy may evoke hypoglycemia, which is sometimes severe enough to cause loss of consciousness as a neurologlycopenic symptom[16]. However, postprandial hypoglycemia following a gastrectomy usually occurs 1.5 to 3 h after food digestion[17]. The present case did not show characteristic postprandial hypoglycemia and the hypoglycemic symptoms improved during the two weeks after surgery, suggesting that this kind of hypoglycemia results from nesidioblastosis rather than from gastrectomy. Additionally, it is assumed that past history of a gastrectomy may have partly aggravated the hypoglycemia in addition to the effect of nesidioblastosis.

When clinical examinations including ASVS suggest nesidioblastosis, a partial pancreatectomy is usually performed. Even if a frozen biopsy confirms the diagnosis of nesidioblastosis, the extent of pancreatic resection remains questionable. A distal pancreatectomy which can control the symptoms of the majority of patients, is well tolerated, and does not induce endocrine or exocrine insufficiency. Recovery after a partial pancreatectomy can remove enough abnormal proliferative tissue to achieve normoglycemia[4,18]. However, some investigators recommend an initial near-total pancreatectomy. Such extensive resections lead to an increased risk of post-surgical diabetes and pancreatic insufficiency. It seems that the best recommendation is a 70%-80% pancreatectomy, administration of diazoxide when hypoglycemia persists post-operatively, and a more extensive resection when previous measures fail[19-21].

In summary, we report here a case of nesidioblastosis of a patient having a history of a subtotal gastrectomy based on a diagnosis of a gastric adenocarcinoma. ASVS can detect a hyperinsulinemic lesion of the distal pancreas. However, sporadic hypoglycemia may occur after surgery, and is refractory to medication. Further study is needed to improve its treatment.

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CASE REPORT

Double ischemic ileal stenosis secondary to mesenteric injury after blunt abdominal trauma

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Abstract

The authors describe a rare case in which blunt abdominal trauma resulted in mesenteric injury with delayed double ischemic ileal stenosis. Abdominal computed tomography demonstrated stenotic ileal loop with mural thickening. At surgery, a double stenotic bowel loop was found adjacent to a healed defect in the mesentery. Histological examination of the two resected segments showed fibrotic and ischemic lesions within the mesentery. Ischemic intestinal stenosis from mesenteric injury should be considered in the differential diagnosis in patients suffering from intestinal occlusion with a history of blunt abdominal trauma.

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Key words: Blunt abdominal trauma; Mesenteric injury; Intestinal stenosis; Obstruction

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INTRODUCTION

In the current treatment of blunt abdominal trauma, conservative management and observation with computed tomography (CT) have become the standard of care for the non-life threatening abdominal lesions. Ischemic intestinal stenosis has been described as a delayed rare complication of mesenteric blunt abdominal trauma. The authors present herein a case of post-traumatic mesenteric injury proved by histological examination, and with double bowel stenosis on CT scan and enteroclysis.

CASE REPORT

A 35-year-old female was admitted after an automobile accident with sudden deceleration. She drove the car and carried the safety belt. The admission check-up showed a cranial trauma and multiple fractures, in particular of the two inferior limbs. She underwent contrast enhanced abdominal CT which showed moderate fluid in the peritoneal cavity attributed to recent bleeding, but no free air, and no parenchymatous, visceral or vascular lesions. She was transferred to the traumatologic unit for surgical osteosynthesis, after which she underwent revalidation treatment.

Six weeks later, she returned to the emergency department with abdominal pain. Altogether, she suffered from four painful abdominal crises with vomiting, described as acute, periumbilical cramps, spontaneously resoluting, compatible with subocclusion or König syndrome.

Abdominal CT showed two ileal loops with a thickened wall and narrowed lumen (Figure 1) adjacent to a mesenteric haziness and minimal fluid in the Douglas. There was no sign of absolute obstruction. An enteroclysis confirmed two intestinal stenosis with rigid ileal loops, one proximal and the other distal (Figure 2).

The patient underwent laparotomy. Two ileal lesions were observed, one located at 180 cm from Treitz's angle, the second one at 60 cm from the Bauhin's valve. Both were scarred ischemic post-traumatic lesions located in front of a mesenteric injury, with thickened ileal wall and narrowed lumen (Figure 3). The two stenosed ileal segments were resected and a latero-lateral anastomosis was performed. Recovery was uneventful. Pathological examination showed mucosal ulceration of the gut located in front of a mesenteric tear (Figure 4), and degenerative infiltration of the mesenteric small vessels, arterioles and veins (Figure 5).

DISCUSSION

Some patients with traumatic intestinal lesion such as perforation or hemorrhage from disruption of mesenteric vessels or parenchymatous tears require immediate surgical intervention. Small and large bowel mesenteric injuries are found in approximately 13.5 % of all patients undergoing

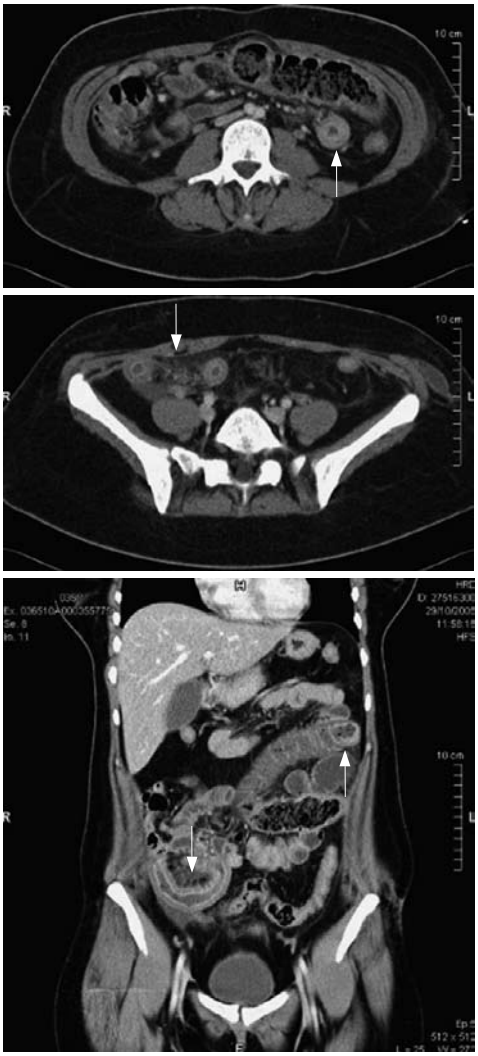


Figure 1 **A**: Transversal abdominal computed tomography, demonstrating two thickened bowel loops with mesenteric haziness (arrows); **B**: Frontal abdominal computed tomography, demonstrating two thickened bowel loops with mesenteric haziness (arrows).

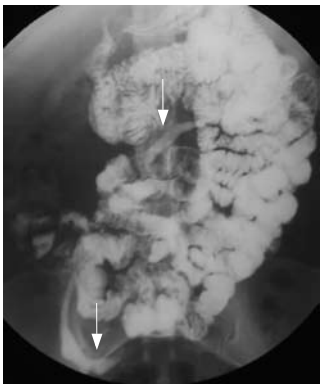


Figure 2 Abdominal enteroclysis showing two intestinal strictures, one proximal, the other distal (arrows).

laparotomy after blunt abdominal trauma[1,2]. In this setting, small bowel injuries are more frequent than colonic injuries, probably due to the factors, including location and lack of redundancy, which prevents formation of closed loops[3]. Contrast enhanced CT should be performed early in patients with blunt abdominal trauma because most significant bowel and mesenteric injuries, as well as associated injuries to other abdominal viscera, may be

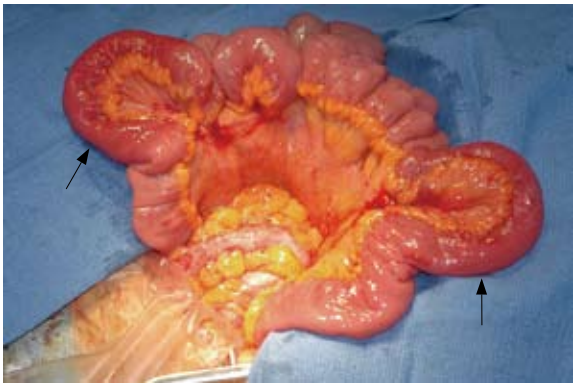


Figure 3 Preoperative picture showing two thickened, fibrotic small bowel segments (arrows) in front of a scarred mesenteric tear.



Figure 4 Inflammation with mucosal ulceration.

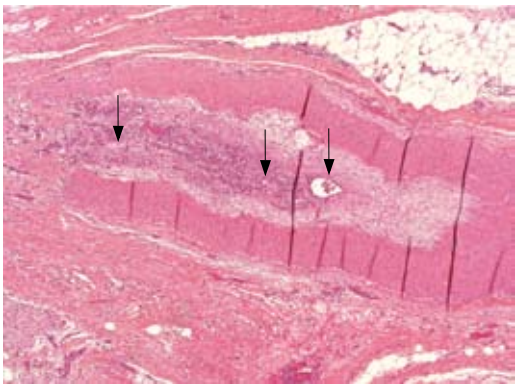


Figure 5 Microscopic section of healed mesenteric injury showing vascular chronic lesion with a repermeabilised venous thrombosis (arrows).

reliably identified. The presence of a moderate to large volume of intra-peritoneal fluid without visible solid organ injury can be an important sign of mesenteric or bowel injury[1], similar to this patient.

On the other hand, mesenteric injury may not cause clinical manifestations[4]. Patients with blunt abdominal trauma are often managed without surgical intervention if there is no sign of bowel perforation or hypovolemic shock. As a result, mesenteric injuries might remain undiagnosed after blunt abdominal trauma.

Small bowel obstruction following blunt abdominal trauma is rare. Three possible causes have been suggested: small bowel perforation[5], localized bowel ischemia[1,4] and mesenteric injury[6]. Mesenteric vascular injury may induce chronic ischemia of the corresponding segment of small bowel, inducing secondary thickening of the bowel wall and intestinal occlusion[1,6]. Post-traumatic bowel lesions may also be found in the colon, mainly on the left

side, specifically the sigmoid colon[7]. The case described herein and its pathological report, further support the mesenteric injury theory of post-traumatic bowel strictures. Most published reports of post-traumatic bowel stenosis described one stricture of the small bowel[8]. The patient reported herein suffered from a double localisation of small bowel lesions, both treated by resection and anastomosis.

Under this particular circumstance, enteroclysis may demonstrate the localization of the stricture, showing a narrowed, rigid loop[9,10]. Abdominal CT may show a bowel wall thickening and a concentric narrowed intestinal lumen, or signs of small bowel obstruction with changed calibre of loops[1]. Although angiography may further demonstrate mesenteric vessel occlusion and provide additional information concerning mesenteric injury[9,11], the authors consider that abdominal CT may provide sufficient information to confirm the clinical suspicion of post-traumatic small bowel stricture and to indicate the need for surgical exploration. Laparotomy with resection of the stenosed segment and primary anastomosis is the appropriate treatment. Early recognition and intervention may prevent prolonged morbidity[10,12].

In summary, a rare case of intestinal occlusion secondary to double small bowel stricture due to abdominal blunt trauma and mesenteric injury was described. This diagnosis should be considered in all patients presenting with abdominal pain some weeks after blunt abdominal trauma.

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CASE REPORT

Crohn's disease complicated by multiple stenoses and internal fistulas clinically mimicking small bowel endometriosis

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Abstract

We report a 31-year-old woman with Crohn's disease complicated by multiple stenoses and internal fistulas clinically misdiagnosed as small bowell endometriosis, due to the patient's perimenstrual symptoms of mechanical subileus for 3 years; at first monthly, but later continuous, and gradually increasing in severity. We performed an exploratory laparotomy for small bowel obstruction, and found multiple ileal strictures and internal enteric fistulas. Because intraoperative findings were thought to indicate Crohn's disease, a right hemicolectomy and partial distal ileum resection were performed for obstructive Crohn's ileitis. Histopathology of the resected specimen revealed Crohn's disease without endometrial tissue. The patient made an uneventful recovery from this procedure and was discharged home 10 d post-operatively. The differential diagnosis of Crohn's diease with intestinal endometriosis may be difficult pre-operatively. The two entities share many overlapping clinical, radiological and pathological features. Nevertheless, when it is difficult to identify the cause of intestinal obstruction in a woman of child-bearing age with cyclical symptoms suggestive of small bowel endometriosis, Crohn's disease should be included in the differential diagnosis.

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Key words: Crohn's disease; Endometriosis; Regional ileitis; Stricture; Internal fistula; Intestinal obstruction; Inflammatory bowel disease

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INTRODUCTION

Endometriosis is a condition of unknown etiology in which proliferation of endometrial tissue occurs at extrauterine sites, including ovaries, fallopian tubes, peritoneum, and gastrointestinal tract[1]. It usually occurs between 30 and 40 years of age and remains active in some patients well into the postmenopausal period[2]. Four to 17% of menstruating women develop endometriosis[3]. Involvement of the intestine with endometrial implants complicates pelvic endometriosis in 17%-37% of cases[4]. Small bowel endometriosis may not be accompanied by pelvic endometriosis[5]. The most commonly affected gastrointestinal sites are the rectosigmoid colon, appendix, and ileum[6]. When the disease involves the small bowel, it usually has a benign course, but in rare circumstances, it may present as acute abdomen[7]. Invasive bowel endometriosis can present as bowel obstruction in an acute, chronic, or intermittent manner[8]. The major cause of obstruction is stricture formation and adhesions, which occasionally mimic Crohn's ileitis or a malignancy in their clinical presentation[9-12].

We report a case of Crohn's disease complicated by multiple strictures and internal fistulas clinically misdiagnosed as small bowell endometriosis, due to the patient's perimenstrual symptoms of mechanical subileus, which required surgical intervention with right hemicolectomy and partial distal ileum resection. To the best of our knowledge, this is the first reported case of complicated Crohn's disease clinically mimicking ileal endometriosis. Moreover, we have been unable to find in the medical literature any report of this particular presentation of complicated Crohn's ileitis.

CASE REPORT

A 31-year-old woman was admitted to our hospital with perimenstrual lower and mid-abdominal pain irradiating to the back and lower abdominal fullness for 3 years, at first monthly, but later continuous, and gradually increasing in severity. She had a history of low-grade fever with chills, which occured monthly during her menstrual periods. The abdominal pain was crampy and associated with

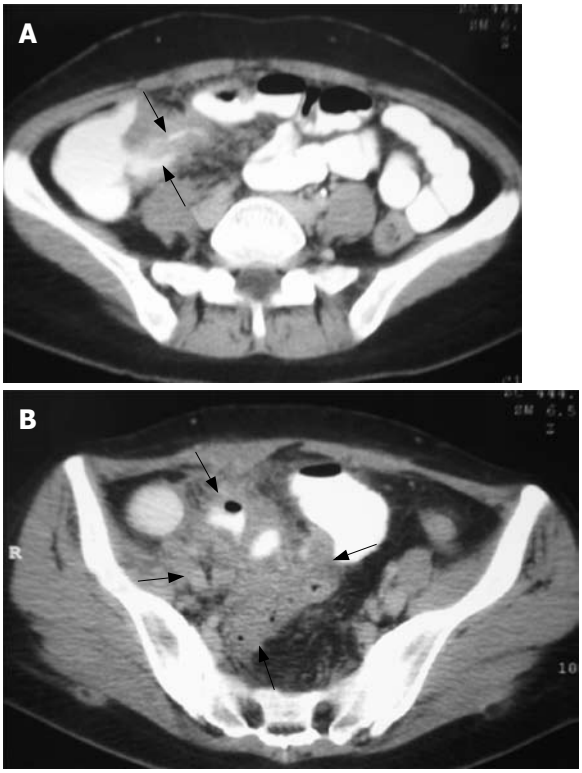


Figure 1 Contrast-enhanced scan showing the mural thickening of terminal ileum (arrows) (**A**), and a complex, predominantly inflammatory mass of large size (6.4 cm × 6.1 cm) (arrows) (**B**).

borborygmi, anorexia, nausea and vomiting, without hematemesis or coffee-ground emesis. The patient had lost 10 kg in weight over the previous 6 mo, and was having two to three semiformed stools per day. She also gave a history of moderate dysmenorrhea and menorrhagia, but no dyspareunia. Her only medication was an oral contraceptive. She denied any history of smoking, alcohol consumption, or illicit drug use. The patient had no history of sexually transmitted disease. She had delivered a healthy baby. There was no family history of inflammatory bowel disease or endometriosis.

Despite no pathological evidence, her gynecologist at a women's health clinic had diagnosed her with small bowel endometriosis, based on interviews and her clinical course. Since only oral contraceptive therapy was started, the symptoms due to mechanical subileus had gradually improved. Although he had no pathological evidence, lack of response to oral contraceptive therapy had encouraged him to perform an exploratory laparotomy 1 mo before she was admitted to our institution for further evaluation and management. At Pfannenstiel transverse laparotomy, extensive adhesion formation and inflammation around the cecum, terminal ileum, sigmoid colon, uterus and the right adnexa were found. The gynecologist was only able to perform an incisional biopsy from the highly inflamed areas. Biopsy results showed only chronic inflammatory infiltrate and granulation tissue. The patient was then referred to us to identify the underlying pathology.

A physical examination on admission revealed a thin and wasted woman weighing 48 kg. The patient was hypotensive with a blood pressure of 95/65 mm Hg and a heart rate of 108 beats/min. The patient's temperature was 37.5℃. The abdomen was soft and distended with right-lower-quadrant tenderness to palpation and fullness. A Pfannenstiel scar was present, but there was no scar tenderness. There was no hepatosplenomegaly or lymphoadenopathy. She demonstrated no arthralgia. Skin rash and erythema nodosum were not found. Rectal examination was unremarkable. There was no perianal fistula. A pelvic examination revealed cervical motion tenderness and right adnexal tenderness. Results of laboratory studies on admission showed: white blood cell count, 28 000/mm^3 with 85.7% neutrophils; hemoglobin, 8.3 g/dL; hematocrit, 24.3%; erytrocyte sedimentation rate, 72 mm/h; C-reactive protein, 26.2 mg/dL; albumin, 3.2 mg/dL; β-human chorionic gonadotropin (β-hCG), 0.1 mIU/mL; carcinoembryonic antigen (CEA), 0.58 ng/mL; and cancer antigen (CA)125, 13.71 U/mL. Urinalysis was in the normal range. Stool studies for ova, parasites, cultures including *Yersinia enterocolitica* and acid-fast bacilli and *Clostridium difficile* toxin were negative. A plain abdominal roentgenogram was consistent with partial small bowel obstruction. Abdominal ultrasonography demonstrated the presence of an inflammatory mass surrounding the terminal ileum and/or appendix, wall thickening and absence of peristalsis in the small intestine, with a small amount of ascites. Abdominopelvic CT scanning was performed with oral and intravenous contrast enhancement. There was a narrowing of the terminal ileum and bowel-wall thickening (Figure 1A). Adjacent to the terminal ileum in the right lower quadrant, a complex predominantly inflammatory mass of large size (6.4 cm × 6.1 cm) was present (Figure 1B). The mass was surrounded by fat stranding, with obliteration of the adjacent psoas muscle fat planes. The appendix was not identified separately from the mass. Colonoscopy with random biopsies from the colon and rectum was unremarkable.

A presumptive diagnosis of mechanical small bowel obstruction was made. The patient was initially treated conservatively with nasogastric suction, intravenous fluids and medication, and responded to this treatment. However, after ingesting a small amount of food she again complained of abdominal pain, and plain radiography once more showed mechanical small bowel obstruction. After improvement with conservative measures and obtaining adequate informed consent, an exploratory laparotomy was performed. At surgery, the proximal small bowel was dilated and the colon was collapsed. There was a small amount of free fluid in the abdomen. No macroscopic evidence of endometriosis was noted at laparatomy. Multiple stenotic ileal loops, adhering firmly to each other, were wrapped as a mass lesion around the cecum, ascending colon, sigmoid colon, right adnexa and uterus. The adhesions were released, and ileal loops were freed from adjacent organs by meticulous sharp dissection. The serosa overlying these areas was congested and hyperemic. The right ureter was completely exposed. Four strictures were noted in the distal 40 cm of the terminal ileum, and three internal fistulas were detected between the terminal ileum and the cecum, between the terminal ileum and the adjacent loop of small bowel, and between the two loops of ileum (Figure 2A and B). The macroscopic appearance

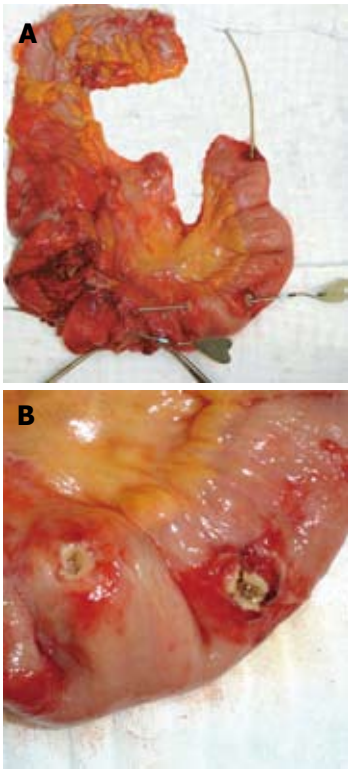


Figure 2 Gross appearance of the resected specimen showing Crohn's ileitis with multiple fistulas probed with instruments (**A**), and ileal segment with two adjacent openings of an internal enteric fistula after separation of adhesions (**B**).

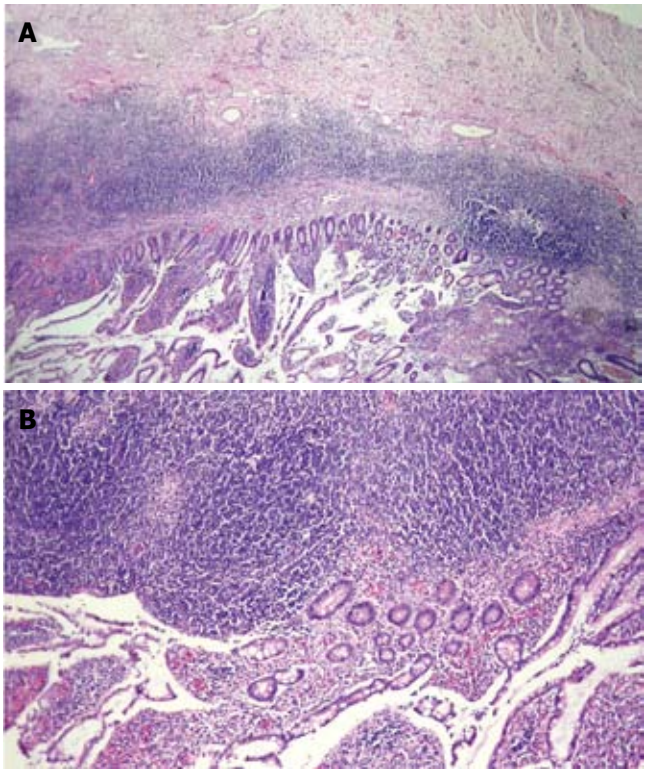


Figure 3 Microscopic examination of the resected specimen revealed transmural inflammatory cell infiltration with crypt distortion (**A**), and transmural lymphoid aggregates (**B**) (HE, **A** × 10, **B** × 20).

was thought to indicate Crohn's disease, but in view of the close relationship of the ovaries, tubes and uterus, an immediate gynecological opinion was obtained. The on-call gynecology registrar did not consider the appearance to be due to primary gynecological pathology, and therefore, a right hemicolectomy and partial distal ileum resection was performed with an end-to-end ileocolonic anastomosis.

On opening the specimen, there was severe narrowing of the terminal ileum and bowel-wall thickening characteristic of Crohn's disease. Ulceration measuring 3 cm in diameter, with an irregular margin, was detected at the level of ileocecal valve. Multiple ulcerations, which ranged in sized from 0.5 cm to 2.5 cm in diameter, were also observed on the oral side of the terminal ileum and adjacent loops of small intestine. The mucosa was edematous, pale and marked with deep linear ulcers, and the bowel wall was thickened. Histological examination of the resected terminal ileum revealed marked ulcers and fissures, with transmural inflammatory cell infiltration (Figure 3A), lymphoid aggregates (Figure 3B), and fibrosis. No endometrial tissue was noted in any segment of the resected specimen. The patient made an uneventful recovery from this procedure and was discharged home 10 d post-operatively. Ileocolic resection led to rapid resolution of the symptoms. She has been asymptomatic for over 1 year after her surgery.

DISCUSSION

Endometrosis is a common condition that occurs most frequently in the pelvis, in association with the ovaries, tubes and pelvic peritoneum, with related gynecological symptoms. The incidence of intestinal endometriosis varies between 3% and 37% of all cases of endometriosis, affecting the rectosigmoid colon in 50%-90%, rectovaginal septum in 10%-20%, cecum in 2%-5%, appendix in 3%-18% and small intestine in 2%-16%[13-17]. Small intestinal involvement is nearly always confined to the terminal ileum, which can be explained by its proximity to the tubes and ovaries. However, the correct diagnosis is often delayed because intestinal endometriosis may masquerade clinically as regional enteritis, ulcerative colitis, appendicitis, ischemic enteritis or colitis, diverticulitis, acute self-limited colitis, irritable bowel syndrome, ileocolonic intussusception, or a neoplasm such as colonic or small bowel cancer[18]. Therefore, many cases of bowel endometriosis have been clinically diagnosed based on interviews and response to hormone therapy.

This case demonstrated multiple strictures and internal fistulas that are frequently observed in complicated Crohn's disease by intestinal contrast, abdominal CT, or at surgery, but we could not make an accurate preoperative diagnosis. There were probably several major reasons for this misdiagnosis. First, our patient presented symptoms of mechanical subileus including intermittent abdominal pain, nausea, vomiting and weight loss, which occured monthly during her menstrual periods. Second, she lacked extraintestinal manifestations associated with Crohn's disease, including stomatitis, aphthous ulcer, colitic arthritis, pericholangitis, erythema nodosum, pyoderma gangrenosum, episcleritis, uveitis, nephrolithiasis, and hydronephrosis. Third, a histopathological diagnosis of the endoscopic biopsy specimen was reported to be negative for inflammatory bowel disease.

Endometriosis of the intestinal tract is a common

disorder that, when symptomatic, may be difficult to diagnose accurately. Intestinal endometriosis has a predilection for the terminal ileum, leads to obstruction, and affects women of reproductive age. Crohn's ileitis must be considered in the differential diagnosis of enteric endometriosis, as with our patient. Other conditions that may cause difficulty in young women with apparent terminal ileal disease include nodular terminal hyperplasia, *Yersinia* enterocolitis, tuberculous enteritis, lymphogranuloma, small bowel lymphoma, carcinoid tumors of the terminal ileum, primary adenocarcinoma of the small intestine, and Behcet's disease[9,10]. Many of these conditions will be diagnosed only with laparotomy.

The distinction of ileal endometriosis from Crohn's disease may be difficult pre-operatively. Indeed, there is a similarity between the two entities in terms of clinical presentation, symptomatology, radiological appearances, surgical and pathological findings. The nature of presentation is mainly dependent on the region of bowel affected for both ileal endometriosis and Crohn's disease. The ileum is the third most affected area, after the rectosigmoid colon and appendix, which is found to be involved in up to 7% of patients with intestinal endometriosis[19]. It usually involves the distal ileum and is limited to the serosa. More extensive and deeper involvement may produce intestinal obstruction by stenosis, kinking or adhesions. Fibrosis may cause puckering and kinking of the mucosa, which results in intestinal bleeding[20]. The presentation of ileal involvement may be an incidental mass found on barium enema or CT, or as a lead point for ileal intussusception. However, Crohn's disease almost invariably affects the gastrointestinal tract. Crohn's ileitis, affecting the ileum only, accounts for 30% of cases. There are three categories of disease presentation in Crohn's disease: stricturing, penetrating, and inflammatory (non-stricturing, non-penetrating disease)[21]. The clinical presentation of our patient with evidence of small bowel obstruction, multiple ileal strictures and internal fistulas at laparotomy suggested Crohn's ileitis, probably due to a combination of inflammatory mass in the right lower quadrant, proximal bowel distention, narrowing of the terminal ileum and bowel-wall thickening.

Endometriosis may present with a wide variety of symptoms that are more commonly associated with other diseases. Gastrointestinal endometriosis is suggested by dysmenorrhea, menorrhagia or perimenstrual symptoms. Intestinal involvement is usually only serosal, not associated with any intestinal symptoms, and only coincidentally noted at the time of open surgery or laparoscopy for gynecological symptoms. Frank intestinal symptoms are usually associated with intestinal obstruction[22]. While intestinal symptoms may occur during or be exacerbated by the menses, this association may not always be present. The symptoms coincide with menstruation in only 18%-40% of the cases in some series[23]. A recurring crampy lower or mid-abdominal pain is the most common presenting symptom for both enteric endometriosis and Crohn's disease. Moreover, right-sided or right-lower-quadrant abdominal pain often precipitates laparotomy or laparoscopy, possibly for the suspicion of appendicitis. Other symptoms that may occur in ileal endometriosis as well as Crohn's disease include diarrhea, constipation, nausea, vomiting, fever, anorexia, and weight loss. We initially considered ileal involvement by gastrointestinal endometriosis because of a history of the relationship between menstruation and presenting symptoms, and also previous surgery for misdiagnosis of enteric endometriosis in our patient.

Small bowel endometriosis is capable of producing both acute and chronic symptoms that can present a diagnostic and therapeutic challenge to the surgeon. Imaging studies may not provide an accurate diagnosis. The radiologic appearances of ileal endometriosis may be so similar to ileal Crohn's disease that a differential diagnosis is not possible. Plain abdominal X-ray or barium enema may be helpful. Contrast studies may demonstrate extrinsic bowel compression, abdominal mass, and bowel stenosis or angulation[24]. Typically, it appears as a long filling defect with tapering margins. Non-invasive radiological diagnostic aids are provided by CT or ultrasonography. Abdominal ultrasonography demonstrates the size, shape and location of lesions, but unequivocal diagnosis is rarely possible. In our patient, ultrasonography demonstrated the presence of an inflammatory mass in the right lower quadrant, and also wall thickening and the absence of peristalsis in the small intestine, with a small amount of ascites. CT is useful for evaluating the small bowel with enteroclysis protocols. The findings of enteric endometriosis on CT include one or more focal masses on the bowel wall or a lesion compressing the colon. Abdominal CT is especially useful when looking for intra-abdominal complications of Crohn's disease, such as abscesses, small bowel obstruction, or fistulas[25]. When thin-section abdominopelvic images with multidetector CT were obtained from our patient, there was a narrowing of the terminal ileum and bowel-wall thickening. It was not certain whether this was due to extrinsic compression or a primary intestinal lesion. Adjacent to the terminal ileum in the right lower quadrant, a complex, predominantly inflammatory mass was also detected on CT. Nevertheless, multiple stenotic ileal segments and small intestinal fistulas associated with Crohn's disease were not seen on CT slices, possibly due to extensive adhesion formation and inflammation. Therefore, we were not able to exclude enteric endometriosis according to the presented radiological findings pre-operatively.

Crohn's disease usually has a distinctive appearance operatively which is not to be confused with the findings in endometriosis, even if multiple site ileal endometriotic deposits are present[26]. Furthermore, in both diseases the surgical intention is limited resection of obstructing lesions, so there is no conflict of interest. An even more difficult, but more important operative distinction is malignant disease, and clearly this is relevant for cecal and ileal lesions, such as colonic or small bowel cancer. At operation, intestinal endometriosis appears as tumors having a glistening gray color on cross section[19]. In our patient, multiple stenotic ileal loops, which adhered firmly to each other, were wrapped as an inflammatory mass around the adjacent organs. The serosa overlying these areas was congested and hyperemic. Multiple ileal strictures

and internal enteric fistulas were detected at laparotomy. Intraoperative findings were thought to indicate Crohn's disease, hence a right hemicolectomy and partial distal ileum resection was the treatment of choice for obstructive Crohn's ileitis.

Both small bowel endometriosis and Crohn's disease are characterized grossly by patchy involvement of the small intestine with intervening, uninvolved skip areas of intestine. Moreover, both conditions may be transmural processes with marked chronic inflammatory changes resulting in the formation of strictures, adhesions, mucosal thickening, mural fibrosis, bowel angulation, stenosis, fibrosis and obstruction. Although transmural involvement by Crohn's disease is the result of chronic insult, strictures and masses in endometriosis result largely from profound smooth muscle hypertrophy around endometriotic foci present in the muscularis propria[27]. Other manifestations of Crohn's disease, such as perianal abscesses or fistulas, and inflammatory pseudotumors involving the cecum, terminal ileum and appendix, have been described in intestinal endometriosis[15]. In rare cases, endometriosis may cause deep fissures and even fistulous tracts, further mimicking regional enteritis. In the presented case, severe mural thickening of terminal ileum, with variable luminal stenosis or stricture formation and entero-enteric fistulas, were gross abnormalities. An approximately 40-cm segment of distal ileum, ileocecal valve and cecum had four strictures and three internal enteric fistulas, with associated serosal adhesions.

Endometriosis may cause many of the histological changes that are associated with Crohn's disease. The endometriotic deposists consist of well-defined endometrial glands embedded in a dense cytogenic stroma[29]. Previous studies have observed chronic mucosal changes ranging from focally branching surface crypts, mildly hyperplastic surface epithelium overlying endometriotic foci, hemorrhagic mottling, ulceration, ulceration with inflammation, occasional neutrophils and eosinophils, or a non-specific inflammatory cell infiltrate, to focal glandular architectural abnormality with edema and siderophages[30]. Chronic inflammatory infiltrates composed of lymphocytes and plasma cells surround the deep crypts, and transmural lymphoid aggregates are also seen. Superimposed acute changes with crypt abscesses and surface ulceration may be noted. The changes are focal in most cases and are flanked by normal, unremarkable mucosa[27]. However, histopathological features used to reach the diagnosis of Crohn's disease in the present case included both gross and microscopic features such as a discontinuous, asymmetric distribution of the lesions, patchy inflammation, ulceration, fissuring, transmural distribution of the inflammatory infiltrate and lymphoid aggregates.

To the best of our knowledge, there have been no other case reports of Crohn's disease complicated by multiple ileal strictures and internal enteric fistulas clinically mimicking small bowel endometriosis. Involvement of the intestinal tract with endometriosis may mimic clinically and pathologically a wide spectrum of diseases including infectious etiology, ischemic enteritis/colitis, inflammatory bowel disease, and neoplasms. The differential diagnosis of Crohn's disease with intestinal endometriosis may be difficult pre-operatively. The two entities share many overlapping clinical, radiological, and pathological features. Nevertheless, when it is difficult to identify the cause of intestinal obstruction in a woman of child-bearing age with cyclical symptoms suggestive of small bowel endometriosis, Crohn's disease should be included in the differential diagnosis.

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CASE REPORT

Huge gastric disopyrobezoar: A case report and review of literatures

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Abstract

We reported a case of huge gastric phytobezoar. The gastric phytobezoar was successfully removed through gastrotomy after two failed attempts in endoscopic fragmentation and removal. Disopyrobezoars could be treated either conservatively or surgically. Gastrotomy or laparoscopical management is recommended for the treatment of huge disopyrobezoars.

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Key words: Phytobezoar; Disopyrobezoar; Stomach

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INTRODUCTION

Phytobezoars are common bezoars in the gastrointestinal tract, including stomach and small intestine[1], but huge disopyrobezoars are rarely seen clinically. We report a case of huge disopyrobezoar (18 cm × 7.5 cm × 7cm), a kind of phytobezoar caused by persimmon, and to present our experience while reviewing the literature.

CASE REPORT

A 47-year-old man presented with complaints of acute aggravated epigastric distention and pain for half a month after one year of persistent epigastric distention and acid regurgitation, which were uninfluenced by ingesting food. There were no obvious symptoms of gastrointestinal obstruction, e.g., nausea or vomiting. The patient had a long history of overindulgence of bitter persimmons from childhood without the history of gastric surgery.

On physical examination, a solid, movable ellipse mass could be palpated in the left epigastrium and the abdomen was soft. Gastroscopical examination showed a giant brown solid gastric bezoar of 15 cm × 7 cm in size (Figure 1), chronic superficial gastritis as well as two erosions at the corner of the stomach, which were pathologically proven to be chronic superficial active membraneous inflammation. Abdominal computerized tomography revealed a mass-like occupational lesion within the stomach (18 cm long and 7 cm in diameter) with air bubbles retained in its interstices and mottled appearance, compatible with the features of bezoars (Figure 2). The patient was then admitted to the department of gastroenterology of our hospital.

On the 3rd day after admission to hospital, endoscopy (*Fujinon EG 250 WR 5*, Fuji Photo Optical Company Ltd, Tokyo, Japan) was used to fragment the huge bezoar with a mouse-teeth clamp and snare, but it was unsuccessful to extract the bezoar despite the help of gastric lavage using sodium bicarbonate ($NaHCO_3$) because the bezoar was too hard and big. On the 4th d, another attempt of endoscopical fragmentation and extraction procedure also failed. On the 5th day, the patient was transferred to the department of general surgery for operation after two failed attempts in endoscopic fragmentation and removal of the gastric bezoar.

Gastrotomy was performed on the patient and a huge grey ellipse bezoar (18 cm × 7.5 cm × 7 cm) was removed from the gastric lumen (Figure 3). A piece of the bezoar obtained during endoscopy was analyzed by infrared spectroscopy, which revealed that 85% of them was composed of tannin and 10% was cellulose, therefore, the bezoar was considered as disopyrobezoar derived from bitter persimmon. The patient was discharged on the 7th postoperative day. During the 6-month follow-up, the patient did not complain of any discomfort and the two concomitant erosions healed uneventfully on medical therapy shown by another gastroscopy.

DISCUSSION

Bezoars are classified according to their composition into phytobezoar, trichobezoar (hair), lactobezoar (concentrated

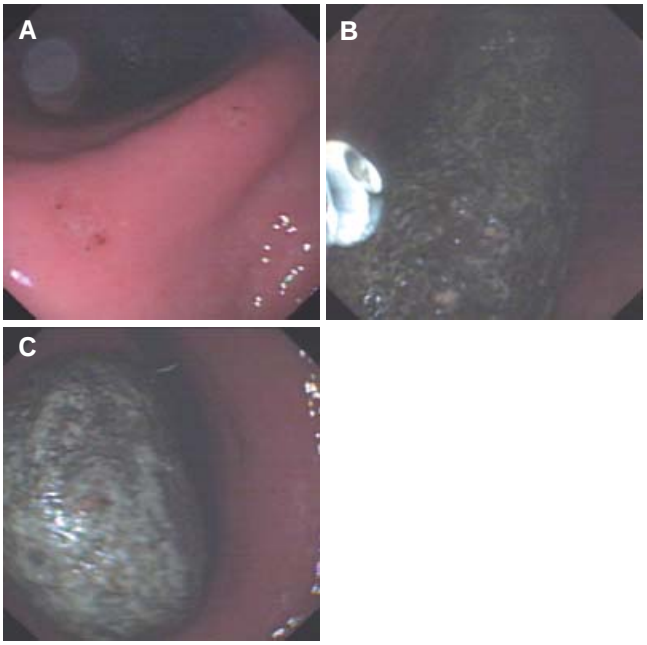


Figure 1 **A**: A huge disopyrobezoar within the stomach shown by gastroscopy, with two erosions at the corner of stomach; **B** and **C**: A huge ellipse disopyrobezoar shown gastroscopically within the stomach.

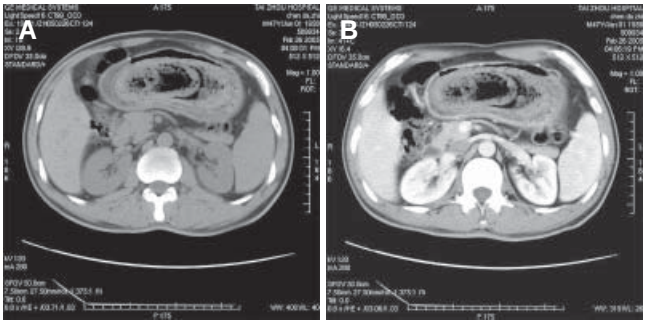


Figure 2 **A**: Unenhanced CT scan showing a huge gastric mass with air bubbles and mottled appearance; **B**: Enhanced CT scan showing the same image as demonstrated on the unenhanced CT scan.



Figure 3 Surgical specimen of gastric disopyrobezoar,18 cm long, 7.5 cm in diameter, with visible brownish persimmon remnants on its surface.

milk formulas), mixed medication bezoar and food bolus bezoar[2]. Phytobezoars are composed of indigestible cellulose, tannin and lignin derived from ingested vegetables and fruits[3].

A disopyrobezoar is a type of phytobezoar caused by persimmons, although it is an infrequent entity in clinic, and not rare in some countries[4]. Disopyrobezoars are generated by over-ingestion of unripe or astringent persimmons containing rich soluble tannin and shibuol especially in free stomach. In the presence of the dilute hydrochloric acid in the stomach, the tannin undergoes polymerization to a coagulum that includes cellulose, hemicellulose, and protein, which is the basis of the bezoar[5].

Disopyrobezoar formation is commonly associated with previous gastric surgery (truncal vagotomy plus pyloroplasty or subtotal gastrectomy plus gastroenterostomy), dental problems, poor mastication, and overindulgence of persimmon[4]. Gastric operations may reduce gastric motor activity and delay gastric emptying. Loss of pyloric function, gastric motility and hypoacidity plays an important role in the formation of disopyrobezoar[6]. Diabetes mellitus and hypothyroidism were also reported as predisposing factors of disopyrobezoar formation because they could delay gastric emptying[7]. Our patient had a long indulgence history of bitter persimmon intake, resulting in the formation of the huge disopyrobezoar.

Clinical manifestations vary with the location of disopyrobezoar from no symptom to acute abdominal syndrome, e.g., epigastric distention, abdominal pain and acid regurgitation. Gastric disopyrobezoars are sometimes associated with gastric ulcer formation. When located in small bowel, they often cause small bowel obstruction (SBO)[1,4], presenting with nausea, vomiting and abdominal distention. Major complications of disopyrobezoars include intestinal obstruction, gastric perforation, gastric ulcer and gastritis[5]. Abdominal pain (49%-100%), epigastric distress (80%), anorexia, vomiting and nausea (35%-78%), and SBO (94.73%) are the main clinical symptoms[8]. Feelings of fullness and dysphagia with weight loss and even gastrointestinal hemorrhage could also be seen[2]. When complicated with SBO, diminished peristaltic sounds, rebounding pain and tenderness, distention, vomiting and abdominal pain could be found clinically, elevated leukocyte count up to 28.000/mm^3 and fever could also be detected[5]. In this study, the patient presented epigastric distention, epigastric pain and acid regurgitation.

As for the radiographic diagnosis, about 50%-75% of the SBO cases could be diagnosed by plain abdominal radiography[9]. CT-scan could demonstrate a well-defined round mass which could be outlined by stomach or the bowel wall and present characteristic internal gas bubbles of bezoars[10]. Abdominal ultrasonography could suggest hyperechoic arclike surface and marked posterior acoustic shadow of the gastric bezoars within the lumen of stomach and small bowel, and barium study could demonstrate intraluminally filling defect as well as mottled appearance of the bezoar. Endoscopic investigations could almost show and confirm all of gastric bezoars[11].

The ultimate goal of the treatment of gastrointestinal disopyrobezoars is the removal of the lesion and prevention of recurrence. Disopyrobezoars can be treated by conservative modalities (gastric lavage, endoscopic

disruption, *etc*.) and conventional surgery as well as videolaparoscopic surgery[4,12]. But disopyrobezoars are often resistant to drug treatment because they are much harder than other kinds of phytobezoars, hence being usually removed endoscopically or surgically. Gastric lavage has been reported for the treatment of disopyrobezoars using $NaHCO_3$ which has a mucolytic effect, and penetration of CO_2 bubbles into the surface of bezoars could digest the fibres of concretion[6]; and most interestingly, a successful nasogastric Coca-Cola lavage treatment for gastric disopyrobezoars was reported[13].

The side effects of conservative treatment of bezoars are gastric ulcer, SBO, hyperosmolar natremia, hemorrhagic pulmonary edema, pharyngeal abscess, endotracheal tube obstruction, esophago-gastric iatrogenic injuries (including perforation, laceration, hematoma and ulceration), vocal cord damage and so on[11].

The first step of endoscopic procedure is to determine whether the pylorus appears anatomically normal and to verify the absence of duodenal stricture before fragmenting disopyrobezoars[4]. If a disopyrobezoar is not too large, it can be extracted by a basket or direct suction. If it is big and pylorus is normal, fragmentation can be performed with large polypectomy snare, electrosurgical knife, endoscopic laser destruction and electrohydraulic lithotripsy as well as extracorporeal shock wave lithotripsy[14]. Once the disopyrobezoar is fragmented, the patient can be treated by combined use of cellulase and metoclopramide, cellulase and papain, water jet and lavage with $NaHCO_3$ as well[4].

In conventional surgery, bezoar removal is commonly done by gastrotomy and/or enterotomy. If complicated with SBO, gastric perforation or gastric hemorrhage, the patients can be treated by gastric and/or intestinal resections. Our patient had a huge solid gastric disopyrobezoar that was extracted by gastrotomy after two failed attempts of bezoar removal by endoscopical fragmentation and dissolution by $NaHCO_3$ gastric lavage. Moreover, the laparoscopic approach may be the treatment of choice when surgery is indicated. When expertise is available, laparoscopy is safe and effective in the management of bezoar-induced SBO and yields superior postoperative outcomes when compared with conventional open approach[15].

In conclusion, disopyrobezoar is a kind of phytobezoar. Plain abdominal radiography, barium studies, ultrasonography, CT-scan and endoscopy are helpful in the diagnosis of disopyrobezoars, while endoscopy can confirm its diagnosis. Disopyrobezoars could be treated either conservatively or surgically. Gastrotomy or laparoscopical manipulation is recommended for the treatment of huge disopyrobezoars.

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CASE REPORT

Anatomical variations of the cystic duct: Two case reports

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Abstract

Anatomical variations of the cystic duct often occur and may be encountered during cholecystectomy. Knowledge of the variable anatomy of the cystic duct and cysticohepatic junction is important to avoid significant ductal injury in biliary surgery. Here, we present two unusual cases with an anomalous cystic duct, namely, low lateral insertion and narrow-winding of the cystic duct. The first case was a 64-year-old man with cholelithiasis and chronic cholecystitis. During surgery, the entrance of the cystic duct was misidentified as being short and leading into the right hepatic duct. Further exploration showed multiple calculi in the right and common hepatic ducts. Cholecystectomy was completed, followed by T-tube drainage of the common and right hepatic ducts. Postoperative T-tube cholangiography demonstrated that the two T tubes were respectively located in the cystic and common hepatic duct. Six weeks later, the retained stones in the distal choledochus were extracted by cholangioscopy through the sinus tract of the T-tube. The second case was a 41-year-old woman, in which, preoperative endoscopic retrograde cholangiopancreatography (ERCP) revealed a long cystic duct, with a narrow and curved-in lumen. The patient underwent open cholecystectomy. Both patients were cured. The authors propose that preoperative ERCP or magnetic resonance cholangiopancreatography (MRCP), and intraoperative cholangiography or cholangioscopy constitute a useful and safe procedure for determining anatomical variations of the cystic duct.

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Key words: Cystic duct; Anatomical variations; Diagnosis; Cholecystectomy

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INTRODUCTION

It is recognized that misidentification of normal anatomy, as well as the presence of anatomical variations, contributes to the occurrence of major postoperative complications, especially biliary injuries[1]. Such injuries can in turn cause significant morbidity and occasionally even mortality. Sound knowledge of the normal anatomy of the extrahepatic biliary tract, as well as the surgical implications[2], is thus essential to prevent these complications. Magnetic resonance cholangiopancreatography (MRCP) is a recently developed technique that allows non-invasive assessment of the biliary tree[3]. Anatomical variants of the cystic duct and cysticohepatic junction that may increase the risk of bile duct injury in biliary surgery are frequently identified with endoscopic retrograde cholangiopancreatography (ERCP), MRCP and percutaneous transhepatic cholangiography (PTC). We highlight two cases of anatomical variations of the cystic duct, in which the abnormality was found during surgery and subsequently confirmed by postoperative cholangiography and ERCP.

CASE REPORT

Case 1

A 64-year-old man was admitted for surgery, with features of acute cholecystitis: right upper quadrant pain, vomiting and fever. Examination revealed a temperature of 38℃ with tenderness in the right upper quadrant. He had previously been investigated for abdominal pain and had abdominal CT 4 years previously, which showed minimal intrahepatic ductal dilatation. Laboratory values showed a normal white cell count, total and direct biluribin was 24 μmol/L and 6 μmol/L respectively, alanine aminotransferase was 80 U/L, aspartate aminotransferase was 38 U/L, alkaline phosphatase was 301 U/L, and amylase was normal. Ultrasonography showed distension of the gallbladder with calculi and a thick wall associated with pericholecystic fluid. Cholelithiasis with chronic cholecystitis was diagnosed, and an open laparotomic cholecystectomy was planned. Upon laparatomy, the liver was normal and the gallbladder was thick-walled with minimal adhesion in Calot's triangle and surrounding

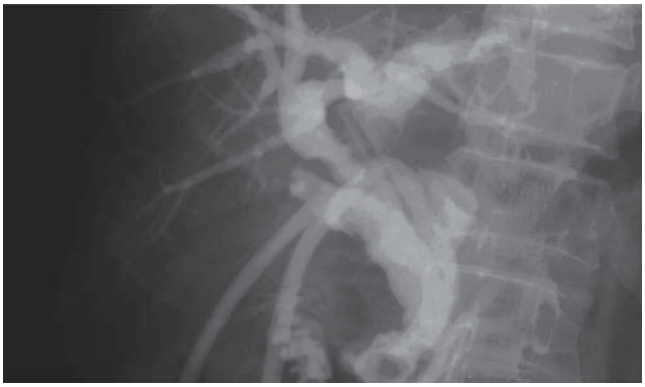


Figure 1 The cystic and left-right hepatic ducts were comparatively long, and the cystic duct opening was located in the distal choledochus with retained stones. Two T tubes were respectively located in the cystic duct and in the common hepatic duct.

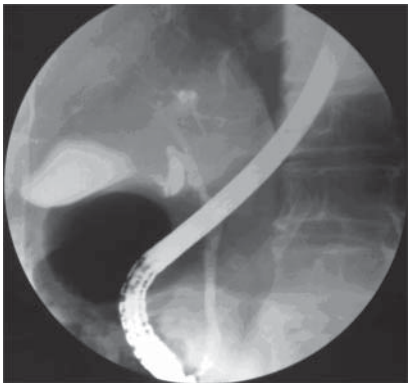


Figure 2 ERCP showing a narrow-winding cystic duct.

tissues. On separation, the entering of cystic was misidentified as being short and entering the right hepatic duct. The cystic duct was clipped and divided. The cystic artery was then identified, clipped and divided. Further exploration showed multiple calculi in the right and common hepatic ducts. Cholecystectomy was completed, followed by T-tube drainage of the common and right hepatic ducts (for postoperative confirmation and documentation). The surgical procedure was followed by abdominal pain, and T-tube cholangiography was requested on postoperative d 14. Cholangiography through a T-tube showed that the cystic and right hepatic ducts were comparatively long. Long cystic duct with low medial insertion into common bile duct was made (Figure 1). The entire bile duct was identified and found to be intact. Six weeks later, the retained stones was extracted by cholangioscopy through the sinus tract of the T-tube. The patient was successfully cured.

Case 2

A 41-year-old woman patient presented with 3-year discontinuous right upper quadrant pain that radiated into her back in keeping with biliary colic, sometimes accompanied with fever and nausea. The pain was aggravated with greasy food. In the course of the present illness, she had received medication for acid peptic disease and parasitic infestations, with no improvement. She had a similar history in the past. Physical examination revealed a soft, non-distended abdomen and no tenderness in the right upper quadrant, without peritoneal signs. Murphy's sign was negative. Her temperature was 37.5℃, and the rest of her vital signs were normal. Laboratory analysis of liver function was normal. US revealed no abnormalities. The patient was observed at rest with continuous gastrointestinal decompression and fluid infusion. Her symptoms improved significantly within 4 d of treatment. However, a few days later, she experienced right upper quadrant pain. ERCP was requested and revealed a long cystic duct with a narrow and in-curved lumen, which was well separated from the gallbladder. The rest of the entire biliary tract was normal without calculi (Figure 2). A narrow-winding cystic duct was created, followed by cholecystectomy. The surgical procedure was followed by an uneventful convalescence until discharge.

DISCUSSION

Anatomical variations of the cystic duct are usually of no clinical significance, occurring in 18%-23% of cases[4]. However, unrecognized variant anatomy can be a source of confusion on imaging studies. In addition, the cystic duct may be involved in a wide variety of both primary and secondary disease processes. The rate of injury varies in the medical literature from 0% to 1%[5]. The following are some of the cystic duct variations found: (1) the cystic and common hepatic duct are in parallel; (2) low confluence of the cystic duct[2]; (3) insertion of the cystic duct in the left and right hepatic ducts, and bifurcation of the left and right hepatic ducts[2]; (4) anterior, posterior spiral types of insertion of the cystic duct on the left side of the common hepatic duct; (5) parahepatic duct insertion into the cystic duct; (6) absent or short cystic duct (length < 5 mm); (7) cystic duct hypertrophy, with a diameter > 5 mm; (8) double cystic duct[6,7]; (9) right hepatic duct emptying into the cystic duct[8]; and (10) hepaticocystic duct[9], a very rare congenital abnormality in which the common hepatic duct enters the gallbladder. The left, right, and common hepatic ducts are all defective, with the cystic duct draining the entire biliary system into the duodenum.

Multiple modalities permit depiction of the normal anatomy, as well as disease processes of the cystic duct, including CT, PTC, ERCP, intraoperative cholangiography and MRCP. Although visualization of the dilated cystic duct is possible with US and CT, the normal-caliber cystic duct may be difficult to detect with these techniques[10]. In our first case, CT demonstrated minimal intrahepatic ductal dilatation, but failed to show low insertion of the cystic duct, as was revealed by surgery. In this case, the low insertion of the cystic duct was misdiagnosed as gallbladder and bile duct calculi. However, in the second case, ERCP showed a long cystic duct with a narrow and in-curved lumen. An anomalous cystic duct was diagnosed before surgery. Anatomical variation is readily identified at ERCP. In clinical practice, if the patient presents with intermittent non-colic right upper abdominal pain, and ultrasound, CT and endoscopy eliminate choledocholithiasis, tumor and peptic ulcer, then a narrow-winding cystic duct should

be considered. ERCP is extremely helpful in diagnosis. Recent studies have demonstrated that MRCP may provide a non-invasive alternative to ERCP and PTC in diagnosis of anomalous cystic ducts[11]. Taourel and colleagues[12] evaluated the accuracy of MRCP in the diagnosis of anatomic variants of the biliary tree in 171 patients. MRCP demonstrated a cystic duct in 126 patients (74%), including low cystic duct insertion in 11 (9%) and a parallel course of the cystic and hepatic ducts in 31 patients (25%). These findings suggest that accurate preoperative assessment is very useful in providing a surgical treatment plan in addition to confirming diagnosis. During cholecystectomy, to avoid biliary tree injury, it is important to identify the common hepatic-cystic duct junction. Misidentification of the cystic duct can lead to postoperative complications. In particular, attention should pay to low medial insertion of the cystic duct because this anatomical variant may lead to misdiagnosis on imaging, and thus affect therapeutic intervention, as was seen in our first case.

A limited literature review of bile duct variation has shown that the aim of most surgeons is to identify whether there are bile duct stones. With respect to the accidental discovery of bile duct variation, it is not the nature of the variation itself but rather the existence of the bile duct variation that is the most important factor in the prevention of bile duct injury. Most injuries to the cystic duct usually occur when it runs parallel to the common bile duct and is encased in a common sheath, so that separation between the ducts is not readily apparent at surgery. T-tube placement in the cystic duct remnant is usually of no consequence; however, there may be a difficulty if retained common duct stones are present, and stone removal *via* the T-tube is attempted. In such cases, access to the bile duct is *via* a tract that enters the cystic duct, and manipulation and extraction must occur *via* the cystic duct across the valves of Heister. Stone extraction is more difficult or may be impossible *via* this route[13]. Suspicion should be raised if the cystic duct is of an unusually large calibre. Intraoperative cholangiography should be used in case of doubt and, in unusual circumstances, cholangiography can be performed *via* the gallbladder to aid in the identification of the cystic duct as well as the common bile duct.

In conclusion, the cystic duct may be involved in a variety of anatomical variations. Diagnostic accuracy relies on a clear understanding of the normal anatomy and anatomical variants of the cystic duct, and imaging features of calculous disease.

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LETTERS TO THE EDITOR

Acute liver failure is frequent during heat stroke

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Abstract

Acute liver failure (ALF) is relatively frequent during heat stroke (HS). This risk must be emphasized, because its incidence is higher than is usually thought. In a recent study by Weigand *et al*, two cases were reported in which liver failure was the leading symptom. We have confirmed their conclusion in a study of 25 cases of HS with ALF, compared with 25 other cases without ALF. Moreover, we observed that hypophosphatemia on admission could predict occurrence of ALF during HS. As for clinical and other biological parameters, phosphatemia should be monitored for at least 3 d in all cases of HS, even when it is thought to be mild.

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Key words: Heat stroke; Hypophosphatemia; Liver failure

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LETTER TO THE EDITOR

We were particularly interested in the excellent study by Weigand *et al*[1], who reported two cases of acute liver failure (ALF) after heat stroke (HS), one during a half marathon [exertional heat stroke (EHS)], the other classical, due to excessive environmental heat, and complicated by multi-organ failure. In addition to severe rhabdomyolysis, each patient was characterized by marked elevation of liver enzymes. Liver injury and ALF are known complications of HS[2]: serum alanine aminotransferase (ALAT) level usually rises within 30 min after HS and peaks within 3-4 d. The risk of ALF during HS must be emphasized, because its incidence appears to be higher than is indicated in this study. Irreversible ALF is rare and may require liver transplantation[3].

We have already reported some of our clinical data[4,5], from a cohort of 50 male subjects (which now consists of 110 cases); all investigated after EHS were confirmed by clinical and biological data, study of muscle metabolism by magnetic resonance spectrophotometry, muscle biopsy for pharmacodynamic tests, and pathology. Twenty-five of these consecutive patients (aged 25 ± 4 years) with EHS and ALF were compared with 25 other EHS patients, who were age-matched but without ALF (ALAT 3563 ± 1313 *vs* 590 ± 742 IU/L; factor V 30% ± 12% *vs* 66% ± 20%; $P < 0.001$ for each). ALF was defined as ALAT > 10 times the upper limit, and coagulopathy (factor V lower than 50%).

Age, body mass index, physical fitness, background, climatic conditions, drug and alcohol intake, clinical manifestations and laboratory findings were analyzed with reference to their prognostic significance in ALF. A logistic regression model was used. In 22 of 25 patients, ALAT level returned to normal within 10 d, but three patients died of ALF. Univariate analysis found that poor fitness ($P = 0.02$), hygrometry > 86% ($P = 0.03$), creatininemia > 160 μmol/L ($P < 0.001$) and hypophosphatemia < 0.5 mmol/L ($P < 0.001$) were significant predictors of ALF. In multivariate analysis, on admission, hypophosphatemia < 0.5 mmol/L was the only independent predictive factor of ALF (RR 3.8, 95% CI 1.1-6.2).

In conclusion, ALF is not uncommon in EHS of which we assert, as Weigand *et al*[1], that this one is an underestimated cause.

This ALF is strongly associated with early hypophosphatemia, of which a value < 0.5 mmol/L is predictive. However, there is no evidence that hypophosphatemia by itself causes important liver dysfunction[6]. Massive liver cell necrosis results from thermal shock, circulatory disruption, endotoxinemia (heat sepsis), high blood concentration of cytokines and acute-phase proteins.

Therefore, in EHS, measurement of phosphatemia, ALAT and factor V should be systematic on admission, and 3-4 d later. Phosphorus supply, usual in an intensive care unit, has not been evaluated in this situation.

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EDITORIAL

Hygiene hypothesis in inflammatory bowel disease: A critical review of the literature

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Abstract

The hygiene hypothesis is thought to be a significant contributor to the growing incidence of inflammatory bowel disease (IBD) around the world, although the evidence for specific factors that underlie the hygiene hypothesis in IBD is unclear. We aimed to systematically review the literature to determine which hygiene-related factors are associated with the development of IBD. Publications identified from a broad based MEDLINE and Current Contents search between 1966 and 2007 on key terms relevant to the 'hygiene hypothesis' and IBD including *H pylori* exposure, helminths, cold chain hypothesis, measles infection and vaccination, antibiotic use, breastfeeding, family size, sibship, urban upbringing, day care attendance and domestic hygiene were reviewed. The literature suggests that the hygiene hypothesis and its association with decreased microbial exposure in childhood probably plays an important role in the development of IBD, although the strength of the supporting data for each of the factors varies considerably. The most promising factors that may potentially be associated with development of IBD include *H pylori* exposure, helminths, breastfeeding and sibship. However, the vast majority of studies in this area are plagued by serious methodological shortcomings, particularly the reliance on retrospective recall of information making it difficult to truly ascertain the importance of a 'hygiene hypothesis' in IBD. The 'hygiene hypothesis' in IBD is an important area of research that may give clues to the aetiology of this disease. Directions for future research are recommended.

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Key words: Inflammatory bowel disease; Hygiene hypothesis; Microbial exposure; Cold chain hypothesis; *H pylori*; Helminths; Measles; Antibiotic; Breastfeeding; Child care

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract[1]. Crohn's disease and ulcerative colitis represent the two most common forms of the condition, with both associated with significant morbidity[2,3]. While the aetiology of IBD remains obscure, it is thought to be the result of a combination of genetic and environmental factors[4]. Although genetic factors including NOD2, IL-23R and ATG16 genes have been implicated in Crohn's disease (CD) and to a lesser degree in and ulcerative colitis (UC)[5,6], they do not account for the striking rise in the incidence of IBD seen over the course of the 20th century[7], raising a strong possibility for an environmental hypothesis in IBD. Only two environmental influences (smoking and appendectomy)[8,9], however, are well established risk factors for IBD, although many others (including oral contraceptives and diet)[10,11] have been proposed to be important in the condition but with inconsistent results[12]. One promising group of environmental factors that may be potentially associated with IBD are those related to the "hygiene hypothesis"[13], which is closely linked to decreased microbial exposure in childhood.

In this review we aim to critically review the evidence for a hygiene hypothesis in IBD by examining factors that are directly linked to microbial exposure, factors that modify the response to infection and proxy measures for microbial exposure. Understanding the role of environmental factors such as these is critical for the possible prevention of IBD in genetically predisposed individuals as well as potentially offering disease improvement to those already suffering with the disease.

THE HYGIENE HYPOTHESIS

The hygiene hypothesis as a potential explanation for IBD comes from observations that the rise in the incidence of IBD, both in developed and developing countries[14,15], has coincided with improvements in hygiene over the 20th century. These improvements in hygiene include access to clean water, a hot water tap, a smaller family size and

thus less crowding, non-contaminated food and hygiene products such as toothpaste[16,17]. There are suggestions however that the rise in allergic and autoimmune disorders[18] over the last century, particularly in developing countries may not be accurate. Based on data from the World Health Organisation it is evident that there is extremely poor coverage of even basic demographic information such as vital registration of deaths[19]. Moreover, so-called Third World economies cannot provide the necessary public infrastructure necessary for diagnosis and reporting of these types of conditions, thus raising doubts as to the importance of a hygiene hypothesis in these disorders.

Nonetheless, Strachan (1989) was the first to link the hygiene hypothesis to the rise in allergic diseases[13]. He reported an inverse relationship between family size and the development of atopic disorders. The hygiene hypothesis is based on the possibility that a child could be overprotected from exposure to common infectious agents in the environment owing to improved hygiene[20]. If the child then comes into contact with a pathogenic infectious agent later in life (delayed exposure), an inappropriate immunologic response is triggered that could lead to the development of an abnormal or ineffective inflammatory process and possibly even IBD.

MICROBIAL EXPOSURE

Improved hygiene is believed to result in a limited exposure to micro-organisms[21]. Such exposure is thought to be necessary in programming the immune system of the gut and mitigating its future inflammatory responses, perhaps even resulting in CD when the immune system is challenged[21]. The underlying premise is that early childhood infection helps to establish the immunological balance between pro-inflammatory Th1 and tolerance-inducing regulatory T cells, preventing the subsequent untoward responses to allergens, microbial or other antigenic stimuli[22]. Thus various childhood circumstances such as day care attendance, presence of siblings, and domestic hygiene-related factors can influence the probability of contracting a "viral infection" at a vulnerable time in immunological development.

We performed a broad based MEDLINE and Current Contents search between 1966 and 2007 on key terms relevant to the "hygiene hypothesis" and IBD including *H pylori* exposure, helminths, cold chain hypothesis, measles infection and vaccination, antibiotic use, breastfeeding, family size, sibship, urban upbringing, day care attendance and domestic hygiene were reviewed. The reference lists from all relevant studies located in this process were then used to trace other studies to provide systematic coverage of relevant studies in this area.

FACTORS DIRECTLY LINKED TO MICROBIAL EXPOSURE

H pylori exposure

Multiple studies since 1994 have found a significantly lower seroprevalence of *H pylori* in patients with IBD compared to both matched controls, and "disease" controls[23-25]. The prevalence of this bacterial infection is lower in CD compared to UC, in the majority of these studies. Potential confounders include the effect of salazopyrine, other 5-ASA compounds, and antibiotics on the carriage and eradication of *H pylori*[26,27]. However, neither salazopyrine nor the other 5-ASA drugs used in IBD reach the concentrations required in the corpus and antrum of the stomach to effect *H pylori* eradication. In addition, studies of IBD patients and disease control groups including patients with COPD[28], indicate that the *H pylori* rates in those individuals exposed to multiple antibiotics are in fact higher compared to those exposed to either no antibiotics or fewer courses.

This reciprocal relationship, similar to that seen between UC and appendectomy[9], has also been linked to subtle changes in disease natural history. Vare *et al*, investigating the relationship between *H pylori* and CD, found that seropositive patients presented at a significantly later age (40 years) compared to seronegative CD patients (30 years, $P < 0.001$)[29]. Modification of phenotype was also identified in a separate study in which seropositive non-smoking CD patients had significantly fewer relapses and a lower risk of bowel resection compared to seronegative non-smoking patients ($P < 0.01$ and $P < 0.05$, respectively)[30].

These observations support a protective role for *H pylori* in both the development and natural history of CD, and need further investigation to determine whether this organism is acting as a surrogate marker of childhood exposure, or may have a more direct and wide-ranging effect on gastrointestinal immune development.

Helminths and IBD

With greater urbanisation and other hygiene practices introduced over the last century has come the decreased acquisition of soil-borne helminths in infants. Helminths are thought to play an important immunoregulatory role in the intestinal flora and as such have been linked to the development of IBD[31]. Firstly helminthic infection is associated with a strong Th2 response, which opposes the Th1 response associated with autoimmune disease and CD[32-34]. Secondly, chronic infection with these organisms may generate a network of regulatory T (Treg) cells that secrete transforming growth factor (TGF)-B and interleukin (IL)-10[33,35]. These cytokines may not only regulate aggressive Th1 responses but also control heightened Th2 responses that contribute to chronic allergic diseases. While there is a wealth of data from animal models to support an immunological role for helminths[32-34] there are limited data to confirm these pathways in the human gut. Moreover helminths infection may bring other anti-colitis mechanisms into play, including increases in mucus and water secretion into the gut lumen[36,37], which may influence the interaction between gut bacteria, their products, and a diseased epithelium, as well as impacting on intestinal motility. Helminths may affect microbial ecology[38] and the neuroendocrine response[39], but none of these factors however have been assessed in human studies. While preliminary results

from human trials in IBD patients using *Trichiuris suis*, or pig whip worm, demonstrate clinical efficacy[40] and no significant allergic disease post infection[41]. Long-term data, particularly after repeated exposure however are urgently needed to confirm these results. Thus, helminths may offer a novel therapeutic avenue in IBD patients, but much more research is needed to fully understand the mechanism behind any potential human association.

Cold chain hypothesis

The cold chain hypothesis shows temporal and geographical coincidences between the development of the refrigerator and the outbreak of Crohn's disease[42]. It is also possible that external factors related to the cold chain hypothesis including machine maintenance procedures, food conservation habits and the availability of electricity may also explain these trends[42]. The potential link between the refrigeration of food and Crohn's disease is *via* exposure to psychrotrophic bacteria with pathogenic properties such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium botulinum* and *Bacillus cereus* which are bacteria that are capable of surviving or developing at low temperatures[43]. Some of these bacteria have been identified in CD lesions[44,45]. A recent study by Forbes and Kalantiz (2006) found that the average age of having a fridge was more than 4 years earlier in older IBD patients than in age-matched controls, a difference that was significant[46]. While a greater exposure of the gastrointestinal tract to psychrotrophic bacteria in the early years of life may be a contributory factor to the development of Crohn's disease it is not yet considered to be an independent risk factor for the disease.

Childhood infections

An infectious aetiology has been proposed for IBD based on studies finding a significantly higher frequency of infectious events e.g. gastroenteritis during the first 6 mo after birth especially among future cases of CD[47,48]. A questionnaire-based study examining both adults and children with IBD have reported an association between the incidence of gastroenteritis and diarrhoea in infancy and the later development of CD[49]. However, an international multicenter study found no difference in the frequency of gastroenteritis in patients with IBD versus control subjects[50]. While numerous infectious agents such as paramyxoviruses[51] and Mycobacterium paratuberculosis[52] have been implicated in CD without consistency[53], it is the measles virus that has received the most attention.

The evidence on whether the measles virus increases the risk of IBD has come from several sources. The first is based on the biological plausibility of a measles virus in IBD. It is hypothesized that the measles virus is able to persist within the mesenteric endothelium creating an inflammatory reaction characteristic of CD[54]. While several studies have identified the presence of the measles virus in tissue samples[55,56], this finding has not been confirmed in studies using more sensitive methods such as the polymerase chain reaction (PCR)[57,58].

Another group of studies have examined the association of *in utero* and perinatal measles exposure and the development of IBD. Ekbom *et al* (1996) reviewed the hospital charts from 25 000 deliveries at a Swedish hospital between 1940 and 1949[59]. They found 4 cases of measles infection during pregnancy and of these, three of the offspring had developed CD. This study however suffered from some important biases including the selection of a period of time when it was known that in two cases of measles in the mother were followed by CD in the offspring. Moreover the diagnoses of maternal measles was based on clinical criteria only and the retrospective design makes it impossible to determine whether it is the measles exposure or some other factor during pregnancy that led to the subsequent development of CD in these children. Moreover studies from Denmark[60] and the UK[61] have failed to detect any cases of IBD after exposure to measles virus *in utero*. A history of perinatal infection has been reported to increase the risk of developing CD by five times[62], although the risk for IBD from measles infection specifically is much lower at 1.5 and is not significant[63]. Moreover Haslam *et al* (2000) did not find an increased risk for Crohn's among children born in years with high measles incidence rates versus children born in other years[64].

In summary the evidence supporting an association between measles infection in terms of in situ detection of measles virus, in utero and perinatal exposure and ecological evidence is discordant, but does not appear to be a causative factor in this condition.

FACTORS MODIFYING THE RESPONSE TO INFECTION

Childhood vaccinations

Childhood vaccinations have the ability to alter the maturation of the intestinal and systemic immune system and as such have been implicated in IBD[65]. Thompson *et al*, 1995) were the first to raise the possibility of a link between measles vaccination and IBD[65]. In a cohort analysis of 3545 individuals in the U.K. they showed that individuals with a history of exposure to measles vaccination were 3 fold and 2.5 fold more likely to develop CD and UC, respectively, compared with unvaccinated controls. Methodological shortcomings such as selection of participants from different populations, differential loss to follow-up between the two cohorts, and different ascertainment of outcome by exposure category, however, all dampened the strength of their findings. Subsequent cohort studies were also unable to confirm these initial findings[66,67].

Further controversy was fuelled when Wakefield *et al* reported 8 out of 12 children with non-specific colitis had symptoms attributable by the parents to the measles, mumps and rubella (MMR) vaccination[68]. Several issues related to selection and recall bias, no control group and lack of a clear case definition raised concerns as to the validity of these claims. In response to public concern, a case control study from the Vaccine Safety Datalink Project was conducted to address the link between MMR and risk of IBD[69]. Cases were 142 patients with chart confirmed IBD born 1958 to 1989 who were compared

to 432 controls matched by birth year, gender and health maintenance organisation. There was no evidence that either monovalent measles vaccine or the combination of MMR vaccination was associated with risk of IBD. One study found a significantly increased risk for CD with increasing age, but this was based on three children vaccinated at 2 years or older[67].

Evidence from ecological studies has generally failed to find a link between the incidence of measles vaccination and IBD[70-72]. The incidence of CD has been reported to be increasing since the 1940's, twenty years before the introduction of the measles vaccine.

Although this is a controversial area of research, the findings from well-designed studies do not support a link between vaccines containing measles and the subsequent development of IBD.

Antibiotics and development of IBD

The critical relationship between the human gut and its microflora has been highlighted in the field of IBD, particularly with the development of molecular models of the disorder such as the IL-10 knockout mouse[73]. These models clearly indicate that IBD does not develop in a germ-free environment. The role of gut bacteria has been further underlined by recent genetic discoveries that point to disturbances in the recognition and handling of bacteria in CD[74].

Antibiotics can potentially influence and disturb this relationship, and therefore have been the subject of several case-control studies in IBD[50,75,76]. In addition, some authors have pointed to the temporal relationship between the introduction of antibiotics on a wide scale after the Second World War, and the significant increase in the incidence of IBD seen in the second half of the last century[77].

There are a limited number of studies that address the role of antibiotics in the aetiology of IBD. Two small early studies were both heavily influenced by recall bias and the potential for reverse causation[50,75]. These found a positive association between antibiotic use and CD. In view of the potential lead-in time between onset of symptoms and actual diagnosis of CD, it is perhaps not surprising then that there may be increased prescriptions for antibiotics given to CD patients in the 1-2 years prior to diagnosis. With this confounder in mind, Card *et al* analysed the General Practice Research database (GPRD) in the UK to pull out prospectively collected data on CD cases and controls[76]. This study assessed antibiotic usage, other medication use, and smoking status as well as addressing the above issue of lead-in time, by analysing data 2-5 years pre-diagnosis. The investigators found that 71% of 587 CD patients were prescribed antibiotics 2-5 years prior to diagnosis compared to 58% of 1460 controls (Adjusted OR 1.32 [1.05-1.65], $P < 0.001$). Although there was no obvious confounding by gender, age, or smoking status, there was a lack of specificity with respect to the type of antibiotic prescribed, and the finding of associations with other drug groups including those for neurological conditions and oral contraceptive pills. This indicated that the association with antibiotics may not be causal. However, the strong interaction between cases and use of tetracyclines, particularly in those subjects with no prior gastrointestinal symptoms nor gastrointestinal medications, was intriguing and may provide some guidance as to future studies in this area.

Breastfeeding

The role of infant feeding practices may play a role in the development of IBD by affecting the early exposure to dietary antigens. Some of the important differences between human breast milk and infant formula include immunoglobulins (IG's), lactoferrin, lysozyme, growth factors, allergic factors, carnitine and DHA & ARA. For example Lactoferrin is an iron-binding protein found in human milk but not in formulas. It limits the availability of iron to bacteria in the gut and alters which "healthy" bacteria will thrive in the gut. It has a direct antibiotic effect on bacteria such as *Staphylococcus spp.* and *E. coli*[78].

The majority of the evidence supports a protective effect of breastfeeding in UC and CD[79-81]. A recent study based on an Italian population of 819 IBD patients suggested lack of breastfeeding to be associated with an increased risk of both CD and UC[82]. These results were supported by paediatric studies in Canada and the United States that found paediatric CD patients were less likely to have been breastfed[83,84]. Gilat *et al* performed a 14-center, 9-country study looking at more than 400 IBD patients whose disease started prior to 20 years of age and who were younger than 25 years at the time of the study[50]. These investigators found no significant difference between patients and control subjects in the frequency of breastfeeding as well as consumption of cereal or refined sugar during infancy.

A major problem with studies in this area are their retrospective design and dependence on patient or maternal recall to complete questionnaires. Moreover no studies have examined the potential confounding effects on the development of IBD of environmental factors such as maternal exposure to endocrine disrupting chemicals and the subsequent levels in human breast milk[85]. Further prospective studies using larger sample sizes is necessary to strengthen the validity of these observations.

PROXY MEASURES OF MICROBIAL EXPOSURE

A range of factors including family size, sibship, birth order, urban upbringing, day care attendance and domestic hygiene such as presence of a hot water tap and flush toilets have been examined in IBD studies as a proxy marker of environmental exposures in early life. Table 1 summarises the methodologies of key studies in this area. It can be seen that there is a heavy reliance on adult case-control retrospective designed studies and while many variables have been studied; only a few are significantly consistent.

Family size/ sibship/ birth order

Family size can be used to indicate the level of over-crowding in a home, which has been associated with poor

Table 1 Key studies examining the association between IBD and proxy measures of the 'hygiene hypothesis'

Author	Sample	Study design	Variables examined
Gilat, 1987[50]	14 centres from 9 countries UC = 197; CD = 302 Diagnosis before 20 yr, all patients < 25 yr old Age-sex match controls	Case control Questionnaires	Siblings, birth order, breast feeding, infection, eczema, family history, vaccination, pregnancy factors
Rigas, 1993[84]	New York, USA Diagnosed between 1986-1990 CD = 68; UC = 39 Pediatric gastroenterology controls = 202	Case control Questionnaires	Sibship size, maternal age at birth, month of birth, breastfeeding, maternal smoking one more
Gent *et al*, 1994[92]	UK UC = 231; CD = 133 16-87 yr Age-sex matched controls from same general practice as cases	Case control Home interview	Housing in infancy, presence of a lavatory, hot water tap, separate bathroom
Duggan *et al*, 1998[93]	UK Consecutive weekly attendees at IBD clinics UC = 213, CD = 110 (aged over 15 yr) Aged-Sex match controls from hospital patients undergoing elective surgery	Case control Questionnaire	Previous surgery, childhood domestic circumstances before age 11 yr (heating, day care, toilets, fixed bathroom, bedroom sharing)
Sicilia *et al*, 2001[87]	Spain Gastroenterologists recruited incident cases of CD patients aged 10-79 yr = 103 Outpatient clinic patient controls matched for age, sex and urban/rural.	Case control Population based study Structured interview 95% response rate	Number of persons in home, number of bathrooms, availability of hot water
Montgomery *et al*, 2002[88]	Swedish inpatient register UC = 15 823; CD = 12 668 Controls from Swedish Census, Births & Deaths Register: age and location matched 79 546; 63 035, respectively	Case control Swedish Multi-generation register linking cases and controls with family history information	Siblings
Feeney *et al*, 2002[16]	UK (Grew up in UK) 16-45 yr CD = 139; UC = 137 attendees at Gastroenterology clinics	Case control General hospital Questionnaire	Hp seroprevalence, family, hot water tap, nursery attendance, indoor toilets, swimming pool use, number or cars, number of house moves, urban/rural location, pets
Hampe *et al*, 2003[89]	Germany Consecutive IBD patients identified from German Crohn's & Colitis association = 2351 Controls unaffected first degree relatives = 3364	Case controls Questionnaire	Availability of tap water, toilet, central heating, siblings and community size
Amre *et al*, 2006[22]	Canada Newly diagnosed CD < 20 yr old = 194 Orthopaedic patients controls matched for timing of diagnosis & area of residence	Hospital based Case control Structured questionnaires - mothers mostly answered for patients	Siblings, breastfeeding, day care, place of residency, hot water tap, toilets, number of inhabitants, number of rooms, availability of private bed, personal hygiene, pet ownership, infection, smoking history

hygiene and potential exposure to infection[86]. A small family size and thus a less propensity for exposure to infection has been associated with a lower risk for IBD[86]. Amre *et al* (2006) created a 'crowding index' which refers to the number of rooms in the home divided by the number of inhabitants living in the home and found the mean 'crowding index' to be lower in cases than controls and thus protective against CD[22]. No differences however were found between the numbers of people residing in the home during infancy (< 18 years) in IBD patients versus controls[87], although this study had limited power.

Siblings may influence the development of IBD by altering exposure patterns to microorganisms in early life by affecting acute manifestation of infection by influencing age of transmission and severity[88]. In a large Swedish case control study, Montgomery *et al* (2002) found having older siblings increased the risk for UC, even after controlling for multiple births, sex, maternal age, year of birth, region and socioeconomic class. This may be because older siblings risk exposing younger siblings to infection at a higher dose or at an earlier age. In contrast, having younger siblings was significantly associated with a decreased risk of CD. This effect was most pronounced when younger siblings were born within two years of the patient's birth. It is hypothesised that having younger siblings may prolong or re-expose the older sibling to the presence of micro-organisms, conferring some protective effect against CD through appropriate priming for mucosal immune function[88]. These findings however have not been replicated in smaller studies[22].

Birth order as a potential marker for the hygiene hypothesis in IBD has been examined in several studies with inconsistent results[50,89]. For example, Hampe *et al* (2003) found birth later in the sibling order was significantly associated with a lower risk for IBD (included patients with CD, UC and indeterminate colitis)[89]. This is because first-born children are usually exposed to infection later in life than younger siblings who come into contact with common viral and bacterial infection through older siblings. Rigas *et al* (1993) however did not find birth order to be significantly associated with either disease based on medical records[84].

Urban upbringing

It has been suggested that an urban upbringing represents a more "hygienic" childhood compared with a childhood spent on a farm or a rural location. Several studies report that a higher incidence of IBD in urban as opposed to rural areas[90,91]. Others however have failed to replicate this finding[16,87].

Day care attendance

Day care attendance in young children has the potential for exposing children to greater infections. Amre *et al* (2006) in a hospital- based case control study found day care attendance in the first 6 mo of life to be associated with an increased risk for CD[22]. Feeney *et al* (2002) however found no significant difference between IBD cases and controls in regards to pre-school attendance[16].

Domestic hygiene

Several domestic hygiene-related exposures have been investigated in relation to IBD. This is because good domestic hygiene may protect an individual from exposure to a full range of agents that programme the immune system in the gut during infancy, causing an inappropriate response, perhaps even CD, when exposure occurs at a later age[92]. Gent *et al* (1994) found CD to be 5 times more common in IBD patients whose first house had a hot water tap and a separate bathroom, but no association was reported for mains drainage or access to a flush toilet[92]. Moreover domestic hygiene variables were not associated with UC[93]. A similar finding was reported by Duggan *et al* (1998) who found availability of hot water before age 11 to be significantly associated with CD but not UC[93]. Others however have failed to find a significant association between availability of hot water, toilet facilities, bed sharing, source of drinking water and number of bathrooms and risk for CD[22,50,89] but some of these studies did not evaluate controls that were representative of the case population or had power problems[50]. Pet ownership[22] and animal exposure during childhood[94] have been associated with an increased risk for CD but are not consistent findings[16]. The frequent use of a swimming pool was significantly associated with CD, although the mechanisms behind the association remain unclear and could be more to do with socioeconomic status rather than hygiene related[16]. Most of the studies except one[50], however, rely on the retrospective collection of information *via* questionnaires making it difficult to rule out recall bias (Table 1).

SOCIOECONOMIC STATUS

Earlier studies cited socioeconomic status as an important factor in the development of IBD, in that a higher socioeconomic status was more prevalent in IBD patients versus controls[95,96]. A higher socioeconomic status may also confer less household crowding and better domestic hygiene (hot tap) and thus increase the risk for CD by limiting exposure to micro-organisms[86]. As hygiene practices have improved over the course of the 20th century, socioeconomic status has become less important in IBD. In a recent study by Bernstein *et al* (2001) no differences in socioeconomic status were found between IBD patients and controls[97].

FUTURE DIRECTIONS

Based on a systematic review of the literature it is evident that there is a strong need for future studies in this area to adopt a prospective design in a paediatric sample to avoid the pitfalls associated with retrospective studies such as recall bias. This is crucial as many factors proposed to be important in the hygiene hypothesis such as breastfeeding date back to birth and infancy. Population based studies are also important to overcome problems inherent with selection biases which may be present in the many patient based studies that dominate this area. The evidence suggests that it is also important to look at Crohn's disease and ulcerative colitis separately since exposure risks do not appear to be the same for both disorders. The need to establish and validate better markers for hygiene related factors is crucial as it is impossible to rule out the possibility that the relationship between IBD and a hygiene related factor such as sanitation for example is not an indirect effect that may serve as a mechanism for other yet unknown lifestyle factors. Future research should also be directed towards trying to understand the potential mechanisms underlying associations between hygiene related factors and IBD in the hope of providing clues to the aetiology of this disease.

CONCLUSION

IBD is a growing disease that is costly to the individual and society. The underlying premise of the hygiene hypothesis is that decreased microbial exposure in childhood may lead to the subsequent development of IBD appears to be plausible, although the strength of the evidence supporting this varies between many hygiene-related factors. Helminthic infection, *H pylori* exposure, antibiotic use, breastfeeding and sibship represent the most promising factors supporting the hygiene hypothesis in IBD, but carefully designed prospective evaluation is urgently needed.

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OBSERVER

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The spirit of Henry Norman Bethune and gastroenterology

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From the Series Editor: A unique column, The Observer, will be initiated soon in *World Journal of Gastroenterology* (*WJG*). Professor Hugh James Freeman, a gastroenterologist from the Department of Medicine, University of British Columbia, Vancouver, has been appointed as a Series Editor for The Observer. Serving as a forum for both gastroenterologists and/or hepatologists worldwide, Professor Freeman will periodically invite experts in particular research fields to examine important progress made in both Gastroenterology and/or Hepatology. The Series will examine challenging issues faced at the fundamental level from the research laboratory to the bedside. The Observer is an invited editorial and will not accept independent uninvited submissions. For more information, please do not hesitate to contact Professor Freeman at hugfree@shaw.ca.

Abstract

Henry Norman Bethune was a physician and surgeon from Canada. He had a highly impressive medical career in Montreal but did his most important work in China where he cared for soldiers on the battlefield. He died in 1939 and was recognized as a hero, but only much later received recognition in Canada. He was a skilled doctor, both as a physician and as a surgeon. However, he was much more and will serve as an inspiration for this series. He was an innovator, an idealist and a perfectionist. It is hoped that this series will gather expert commentaries on a range of issues critical to the subspecialty from fundamental science to clinical care so that future directions can be defined.

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Key words: Bethune; Gastroenterology; China; Canada; Battlefield surgery

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INTRODUCTION

Developing this new editorial feature presents a challenge, but also an important opportunity. There is a broad range of critical issues that gastroenterologists must deal with on a daily basis. Some relate specifically to fundamental scientific aspects of our specialty as well as clinical elements of "cutting edge" patient investigation, treatment and ongoing care, particularly for chronic illnesses. Several subspecialty journals in gastroenterology, hepatology and nutrition already provide new and updated information, from time to time, on many of these issues. Although being informed about the past and present state of knowledge is very important, there is also an increased need to visualize, even speculate about future directions in all of these related fields. It is hoped that this column will help to share such insights through editorials from individual experts focused on specific areas of our discipline.

Other critical areas include the impact of industry and the role that different global or multinational corporations already have in our subspecialty, especially those involved in the production of new pharmaceuticals as well as biological agents. In addition, there are ongoing changes and developments in endoscopic technology and imaging devices that will continue to impact our highly procedure-driven specialty. Moreover, there are critical concerns related to manpower needs to screen for different forms of cancer. Finally, there are demanding needs in developing nations to provide optimal care in our subspecialty. This journal is unusual in its absence of advertising and will certainly provide a different setting for evaluation of many of these issues. Leading experts in the new frontiers of our rapidly expanding discipline will contribute updates in their field along with their insights. Candid editorial comments on the present state of knowledge, controversies, and, perhaps, their predictions related to the future will be encouraged.

BETHUNE

At McGill University in Montreal, it seemed customary during my student years for each new medical class to

become informed about the important efforts of many outstanding physicians that preceded our own experience. Like many other schools, their photographs and sculptures adorned the hallways of the medical school buildings. This feature of the curriculum probably had many purposes, including the development of a form of local pride for the contributions that these early faculty made to clinical and investigative medicine. Some, intimately linked to McGill University, seemed very impressive, including William Osler and Wilder Penfield. Many in my class, however, were most intrigued with the exploits of a rather curious Canadian physician, Henry Norman Bethune, who had a most amazing career ending with his final months in China. His life was first revealed to us in a low budget black-and-white film. Later, it appeared on an almost annual basis, witnessed by our own class, the result being that it seemed to become indelibly imprinted on our brains. Perhaps, in retrospect, this was done purposefully, before and during our initial clinical exposures and encounters on the wards where these ancestral giants had actually worked and toiled in previous times. We were being inspired.

Bethune was born in Gravenhurst, Ontario in 1890. He entered medical school at the University of Toronto, and then spent time as a stretcher bearer in a field ambulance unit for the Canadian Army in France in 1915. In the interim, his medical studies were temporarily interrupted, but eventually, he graduated in 1916. He was reported to have suffered a severe bout of pulmonary tuberculosis leading to surgical treatment using the technique of surgically-induced pneumothorax. This experience inspired his interest in surgery, particularly thoracic surgery.

He eventually came to Montreal and became a member of the McGill Teaching Faculty where he served on the surgical team at the Royal Victoria Hospital. There, he personally developed over a dozen new surgical instruments, including the Bethune Rib Shearer. Remembered by some for his intellect and surgical inventions, Bethune was also a realist, almost painfully so. In spite of a highly impressive career in Montreal, he became very disillusioned as many of his own patients, especially the poor, often re-developed tuberculosis after treatment. In large part, it seemed to Bethune to be due to their almost immediate return to their former poor living conditions after discharge from hospital. As a result, he developed a persisting passion for socialized medicine and, at this early stage in his career raised the issue of universal health care. As time has shown, however, he was a true revolutionary and, initially, was clearly not popular with his medical colleagues in Montreal. Indeed, in Canada, socialized medicine in its current form did not actually appear for almost another half century.

After visiting the Soviet Union, Bethune joined the Communist Party of Canada in 1935. During the Spanish Civil War in 1936, he worked on the Republican side against the Fascists, organizing the first mobile blood transfusion unit and treating battlefield injuries. In 1938, he joined the Chinese army of Mao Tse-Tung (Mao Ze-Dong) and the Communist Party during the Japanese invasion of China. There, he went to Yenan in 1938 and formed the first mobile army surgical hospital (MASH) unit that was carried on mules. Bethune performed numerous surgeries in the Taihang Mountains. He worked with tradesmen to develop new surgical instruments and trained physicians and nursing staff. Bethune died in November 1939 in Tanghsien (Tang xian County), Hopei, during the Sino-Japanese War, apparently of sepsis reported to have resulted from an accidental wound suffered during a surgical procedure without surgical gloves. In December 1939, Mao Tse-Tung (Mao Ze-Dong) wrote an essay entitled "*In Memory of Norman Bethune*" and concluded:

"...We must all learn the spirit of absolute selflessness from him. With this spirit everyone can be useful to the people. A man's ability may be great or small, but if he has this spirit, he is already noble-minded and pure, a man of moral integrity and above vulgar interests, a man who is of value to the people..."

Although Bethune became well known in China, recognition in Canada came only decades later, even after a medical school had been named in his honor, located in Jilin. In 1976, the Bethune Memorial House, a National Historic Site in Canada, was dedicated in Gravenhurst, Ontario. In 1990, a biographical film entitled "Bethune: The Making of a Hero", starring Donald Sutherland, was produced as a joint venture that included Telefilm Canada, the Canadian Broadcasting Corporation and China Film Co-production. During the same year, Canada and China each issued two national postage stamps, identical in design, to honor Bethune. In 1998, Bethune was inducted into the Canadian Medical Hall of Fame. In 2006, China Central Television developed a 20-part drama series directed by Yang Yang, entitled "Dr. Norman Bethune", reported to be the most expensive Chinese television series ever produced.

LOOKING FORWARD

This new editorial section, The Observer, will be dedicated to Henry Norman Bethune and his important contributions as a Chinese doctor. Bethune became a hero in China, not only because he was a very good physician and a skilled surgeon, but also because he was an innovator, an idealist and a perfectionist. These qualities seem especially important today in gastroenterology. It is sincerely hoped that this new journal column will be able to gather expert commentaries on a range of issues critical to the subspecialty from fundamental science to clinical care. Here, it is hoped that current specialty data can be deciphered and its future directions defined. The spirit of this man must be carried forward.

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REVIEW

Overwhelming postsplenectomy infection syndrome in adults - A clinically preventable disease

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Abstract

Overwhelming postsplenectomy infection (OPSI) syndrome is a rare condition, but is associated with high mortality. However, recognition and clinical management of OPSI is not well established. The prevalence of splenectomy increased recently because it was a clinically effective treatment for hepatitis C virus-associated thrombocytopenia before the introduction of the interferon/ribavirin combination therapy. We reviewed the literature characterizing the clinicopathological features of OPSI and assessed the most effective and feasible administration of the condition. A Medline search was performed using the keywords 'overwhelming', 'postsplenectomy infection', 'postsplenectomy sepsis', 'chronic liver disease', and/or 'splenectomy'. Additional articles were obtained from references within the papers identified by the Medline search. Durations between splenectomy and onset of OPSI ranged from less than 1 wk to more than 20 years. Autopsy showed that many patients with OPSI also had Waterhouse-Friderichsen syndrome. Although the mortality rate from OPSI has been reduced by appropriate vaccination and education, the precise pathogenesis and a suitable therapeutic strategy remain to be elucidated. Protein energy malnutrition (PEM) is commonly observed in cirrhotic patients. Since the immune response in patients with PEM is compromised, a more careful management for OPSI should therefore be applied for cirrhotic patients after splenectomy. In addition, strict long-term follow up of OPSI patients including informed consent will lead to a better prognosis.

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Key words: Overwhelming postsplenectomy infection; Splenectomy; Overwhelming; Postsplenectomy sepsis; Chronic liver disease; Postsplenectomy infection

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INTRODUCTION

Splenectomized patients are a significant infection risk, because the spleen is the largest accumulation of lymphoid tissue in the body[1]. Overwhelming postsplenectomy infection (OPSI) is a serious fulminant process that carries a high mortality rate[2-4]. OPSI cases have been well documented, and more recently the syndrome was reviewed in the literature[1,5,6]. The pathogenesis and risk of developing fatal OPSI remain ill-defined, however, especially in the normal adult host.

Anti-hepatitis C virus (HCV) therapy with pegylated interferon has proven effective for virus clearance in recent years[7,8]. Prior to that, treatment of HCV-associated thrombocytopenia was an important and unresolved problem[9,10]. Splenectomy in patients with hepatitis C cirrhosis is now a safer prelude to antiviral treatment[11-14]. However, protein energy malnutrition (PEM) is a common manifestation in patients with liver cirrhosis, with incidences as high as 65%-90%[15-17], putting them at a higher risk of OPSI, because patients with PEM generally have a reduced immune response. This review describes the clinical features of OPSI and discusses management strategies for patients with OPSI, particularly those with chronic liver disease.

Symptoms of OPSI

King and Shumacker first described bacterial sepsis following splenectomy in infants and children in 1952[18]. It emerged subsequently that a comparable syndrome occurs in asplenic adults REF. Fulminant bacterial sepsis in asplenic patients will be termed OPSI in this review in keeping with common usage in the literature.

Aspecific and mild physical symptoms of postsplenectomy appear in the early stages of OPSI. These include fatigue, colored skin, body weight loss, abdominal pain, diarrhea, constipation, nausea, and headache (Table 1)[6,19]. Pneumonia and meningitis are frequent more severe concomitants. The clinical course may rapidly progress to coma and death within 24 to 48 h, due to the high incidence of shock, hypoglycemia, marked acidosis, electrolyte abnormalities, respiratory distress, and disseminated intravascular coagulation[20,21]. The mortality

rate is 50%-70% despite aggressive therapy that includes intravenous fluids, antibiotics, vasopressors, steroids, heparin, packed red blood cells, platelets, cryoprecipitates, and fresh frozen plasma[2,19,22-24]. The later clinical course frequently mirrors that of Waterhouse-Friderichsen syndrome (WFS), and bilateral adrenal hemorrhage may be found at autopsy[25,26]. The mechanism that connects splenectomy to WFS is unknown but the possible causes of OPSI include loss of splenic phagocytic function, decreasing serum immunoglobulin levels, suppression of lymphocyte sensitivity, or a change in the opsonin system[27,28].

Table 1 Clinical features of overwhelming postsplenectomy infection

Cryptic infection (no obvious focus)
Short, nonspecific prodrome
Massive bacteremia with encapsulated organism
Septic shock with disseminated intravascular coagulation
Marked virulence: 50% to 70% mortality
Death ensues in 24 to 48 h

Overwhelming postsplenectomy infection had been defined as septicemia and/or meningitis, usually fulminant but not necessarily fatal, and occurring at any time after removal of the spleen.

Risk factors

Splenectomized patients are at risk of life-threatening sepsis. Major factors for stratifying risk include the age at which splenectomy occurs, the subsequent time interval from splenectomy, the reason for splenectomy, and the overall immune status of the patient REF.

Infants do not acquire specific antibodies against encapsulated organisms until relatively late in the development of antibody responses. Although OPSI can occur at any age, children are therefore at greater risk of developing the condition, especially those under the age of 2 years[29,30]. However, estimates of the incidence and timing of OPSI vary widely in the literature, and some authors report the greatest risk of developing OPSI in the first two years after splenectomy[31]. Recent reports recommended that all asplenic patients should receive optimal advice and protection against OPSI regardless of the underlying etiology, based on evidence that the increased risk of severe sepsis after splenectomy is lifelong[32,33].

Splenectomy performed for a hematological disorder, including thalassemia, hereditary spherocytosis, auto-immune hemolysis, immune thrombocytopenic purpura, or lymphoma, appears to carry a higher risk than splenectomy performed as a result of trauma[34,35]. In addition, patients with hematological and autoimmune disorders, or lymphoma show impaired immunity, and patients undergoing treatment such as chemotherapy often show decreased serum immunoglobulin levels; both groups may therefore have an increased susceptibility for OPSI.

Hyposplenism occurs when splenic functions are reduced by disease or are absent congenitally or after splenectomy. Howell-Jolly bodies are small, round remnants of the original erythrocyte nucleus. Increased numbers of Howell-Jolly bodies in a peripheral blood smear, although not overly sensitive, can identify the degree of hyposplenism that presents a risk for developing OPSI[2,19,36].

Infecting organisms in OPSI

Outside the splenic circulation, polysaccharide antigens are poorly immunogenic in comparison with protein antigens. This contributes to polysaccharide-coated bacteria evading the immune response and subsequent phagocytosis[28]. Host defenses against bacteria are therefore critically dependent on humoral immunity and production of type-specific antibodies. While liver Kupffer cells clear most well-opsonized bacteria, encapsulated organisms resist antibody binding and are primarily removed by the spleen. Sepsis in asplenic patients can occur with any organism, be it bacteria, virus, fungus, or protozoan, however encapsulated organisms are frequently associated with sepsis in splenectomized patients. Encapsulated organisms such as *Streptococcus pneumoniae* are particularly resistant to phagocytosis, but is quickly overcome in the presence of even a small amount of type-specific antibody[24,37]. Without the spleen, prompt antibody production against a newly encountered antigen is impaired and bacteria proliferate rapidly. Therefore, the risk of invasive pneumococcal disease in patients without a spleen is 12-25 times greater than that in the population at large[38,39]. Invasive disease in the asplenic patient due to such encapsulated organisms as *Streptcoccus pneumoniae* (50%-90%), *Neisseria meningitides*, *Hemophilus influenzae*, and *Streptococcus pyogens* (25%) leads to uninhibited bacterial overgrowth[24,29,33,37].

Prevention of OPSI

Treatment of OPSI is generally aggressive due to the serious nature of the condition and associated mortality. Comprising intravenous fluids, antibiotics, vasopressors, steroids, heparin, packed red blood cells, platelets, cryoprecipitates, and fresh frozen plasma, it may fail to alter the course of this fulminant septic syndrome[25]. Therefore, prevention of OPSI is extremely important for immunocompromised patients who have undergone splenectomy.

Preventive strategies including vaccination and education are also important for splenectomized patients. Functionally or anatomically asplenic patients are at increased risk of infection from encapsulated organisms compared to the general population. Vaccines available for the most common organisms include the 23-valent pneumococcal polysaccharide vaccine, a 7-valent protein-conjugated pneumococcal vaccine, the *Hemophilus influenzae* type B vaccine, and the meningococcal vaccine[28]. The polysaccharide-based pneumococcal vaccine is recommended for all adults at increased risk of pneumococcal infection, and particularly the asplenic patients[40]. The Centers for Disease Control and Prevention in the US (revaccination every 6 years) and the British Committee for Standards in Haematology (revaccination every 5-10 years) recommended revaccination for the prevention of OPSI, at the same time emphasizing the rather frequent need for shorter intervals between revaccinations to keep antibody concentrations at a level sufficient by probability to confer protection[41,42]. Unfortunately, fatal pneumococcal sepsis has been

reported in asplenic, vaccinated patients. It remains advisable, nevertheless, to offer splenectomized patients protection due to their increased risk of developing severe disease and because the vaccine itself poses minimal risk. Jockovich[43] reported no OPSI among patients vaccinated before splenectomy; however, 10.4% of patients who did not receive vaccination developed OPSI. In addition, 5% of patients who were given vaccination after splenectomy developed OPSI. For elective splenectomy, the vaccine should be given at least 2 wk before surgery[43,44].

Finally, patient education represents a mandatory strategy for preventing OPSI. Studies have shown that from 11% to 50% of splenectomized patients remain unaware of their increased risk for serious infection or the appropriate health precautions that should be undertaken[45,46]. Patients should understand the potential severity of OPSI and the possibility of rapid progression. Physicians should inform any new healthcare professionals, including dentists, of asplenic status. In particular, the presence of increased Howell-Jolly bodies on a peripheral blood smear should be highlighted on the laboratory report to inform the patients' physicians of possible hyposplenism[2,19,36], This information and its significance should be in turn relayed to the patient. Moreover, advice for asplenic individuals to be issued with a form of medical alert, such as a card or a bracelet, has two purposes. First, it should provide a constant reminder to the individual of their condition and, second, knowledge of their state might be vital for medical attendants in the event of a medical emergency. This aspect of patient management needs increased attention as only one patient was found to hold such a card. The possibility that OPSI episodes in asplenic patients may have been avoided with improved knowledge cannot be excluded[5].

Future directions

HCV is a major public health problem and a leading cause of chronic liver disease. Various studies have suggested that 3%-20% of chronically infected patients will develop cirrhosis over a 20-year period, and are at significant risk of developing hepatocellular carcinoma[47]. Recent therapeutic trials in well-defined, selected populations revealed that combinations of interferons and ribavirin are more effective than monotherapy for HCV treatment. Moreover, trials of pegylated interferons yielded improved sustained virus response rates with similar toxicity profiles. As with all clinical decisions, selection of patients for HCV treatment requires an accurate assessment of therapeutic risk *vs* benefit, a determination that is complicated by exclusion from registration trials of persons with conditions that might increase risk and diminish benefit. Application of these principles to individual patients can be challenging, and the relative strength of recommendation of treatment varies accordingly. However, HCV is also a possible cause of chronic thrombocytopenia in these patients, by a mechanism that remains unclear. Moreover, treatment for HCV often leads to anemia, neutropenia, and thrombocytopenia. These conditions prevent safe application of treatment in patients with baseline pancytopenia, due to the cirrhosis-related hypersplenism[14]. The various treatments used, including steroids and intravenous immunoglobulin, have generally produced only transient responses[48,49]. Nevertheless, partial splenic embolization and laparoscopic splenectomy may enable anti-HCV therapy to be given. Recently, Hayashi *et al*[14] reported that splenectomy for severe thrombocytopenia with the intent to apply interferon therapy can be beneficial. It is therefore feasible that splenectomy prior to anti-HCV treatment for HCV-related chronic thrombocytopenia will occur more frequently in the near future. In addition, physicians should follow the occurrence of OPSI in these patients not only during the treatment for HCV clearance but also for the entire follow-up period.

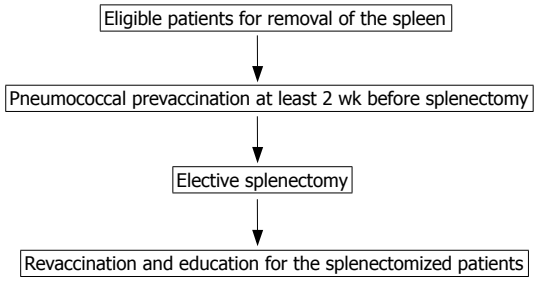


Figure 1 A proposed prevention strategy for splenectomized patients in management algorithm.

CONCLUSION

The spleen is crucial to the host response to infection by clearing polysaccharide-encapsulated bacteria. This response involves the clearing of pathogens from the bloodstream as well as the rapid production of specific antigens. Splenectomy results in an increased risk of septic complications associated with a high mortality rate, the most serious being the development of OPSI. Nonspecific symptoms, including nausea, vomiting, fever, and unconsciousness, followed by a rapid progression to coma and shock characterizes OPSI, and it is treated aggressively.

Eradication of HCV with the recently introduced therapy of pegylated interferon and ribavirin would be particularly beneficial in those with advanced liver fibrosis or cirrhosis. Splenectomy might be useful also to raise platelet counts so that pegylated interferon-based therapy can be performed safely in patients with cirrhosis due to HCV. Prevention strategies such as vaccination and education are also potentially important parts of the strategy for splenectomized HCV patients (Figure 1). Finally, reduction of the immune response in patients with chronic liver disease and/or PEM necessitates careful follow up of splenectomized patients with chronic liver disease to monitor the appearance of OPSI.

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REVIEW

Adenoviral gene therapy in gastric cancer: A review

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Abstract

Gastric cancer is one of the most common malignancies worldwide. With current therapeutic approaches the prognosis of gastric cancer is very poor, as gastric cancer accounts for the second most common cause of death in cancer related deaths. Gastric cancer like almost all other cancers has a molecular genetic basis which relies on disruption in normal cellular regulatory mechanisms regarding cell growth, apoptosis and cell division. Thus novel therapeutic approaches such as gene therapy promise to become the alternative choice of treatment in gastric cancer. In gene therapy, suicide genes, tumor suppressor genes and anti-angiogenesis genes among many others are introduced to cancer cells *via* vectors. Some of the vectors widely used in gene therapy are Adenoviral vectors. This review provides an update of the new developments in adenoviral cancer gene therapy including strategies for inducing apoptosis, inhibiting metastasis and targeting the cancer cells.

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Key words: Gastric cancer; Adenovirus; Gene therapy; Vector; Apoptosis; Metastasis

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INTRODUCTION

Gastric cancer is one of the most common malignancies in the world with an estimated 934 000 cases reported globally in 2002[1], and the second most common cause of death from cancer. The prognosis of gastric cancer is poor with an estimated relative 5 years survival rate of less than 20%[2].

In Iran, 26.1% of cancers reported in 2002 in men and 11.1% of cancers in women were due to gastric cancer. Gastric cancer is the first leading cause of Cancer related death in Iranian men and the second in Iranian females. The incidence rates of gastric cancer in Iran are well above the world average; it is the fourth common cancer in the world however it is the most common malignancy in Iran[3]. Comparing gastric cancer rates with the data of 30 years ago, shows that the overall incidence of gastric cancer in Iran has gradually increased over the years[4]. This is in contrary to the global trend which has seen a steady decrease in the incidence and mortality rates for gastric cancer.

The current best approach for treating gastric cancer is complete surgical removal of the tumor with the adjacent lymph nodes however the efficacy of this therapeutic approach as well as hormone, radio and chemotherapy is very limited. Thus new therapeutic approaches are needed[5,6].

Gastric cancer is a genetic disease developing from a multi-step process[7]. Single or multiple mutations in genes related to growth control, apoptosis, invasion and metastasis form the molecular genetic basis of malignant transformation and tumor progression[8], so a better understanding of the molecular basis of tumor host interactions leads to significant progress in the development of new therapeutic agents. Based on this theory, cancers can be curable if the regulating mechanisms are reintroduced to the cancer cells; this can be achieved using gene therapy, which is an emerging alternative for treatment of cancers[9,10].

In gene therapy vectors are used to insert new genes into cells, or to switch off existing genes[11]. Some of the most efficient vectors used for gene therapy are viral vectors, as they naturally insert their genes into cells, they are well adopted and equipped for such a task. In recent years adenoviruses have been extensively used as vectors to deliver foreign genome into mammalian cells. Adenoviruses have certain features, which make them attractive vectors for gene transfer to target cells. Some of these characteristics include their ability to infect a broad range of cell types, including dividing as well as non-dividing cells, the ease with which adenovirus genome can be manipulated, and the ability to obtain high titers. To achieve an adenoviral vector, foreign cDNAs are inserted into the adenovirus genome, resulting in recombinant adenoviruses containing the gene of choice.

Cancer gene therapy has 3 essential goals: the first is

to suppress cancer growth and induce apoptosis in cancer cells, the second goal is to inhibit metastasis of malignancy to other sites and finally the effects of gene therapy must be limited to cancer cells. These goals can be achieved using some strategies, which we will further explain below[12-15].

TUMOR SUPPRESSOR GENES AND APOPTOSIS INDUCING GENES

As mentioned earlier, one of the ways for stopping cancer growth is to reintroduce the regulating mechanisms into cells, these mechanisms are supervised in the first place by tumor suppressor genes, so by inserting these genes into cells, tumor growth can be suppressed. Reintroduction of tumor suppressor genes also has the additional advantage of making targeted cells susceptible to chemotherapy[16].

The most famous tumor suppressor gene is P53, which is mutated in 60% of gastric cancers and in almost all other cancers[17]. Introduction of the p53 gene *via* a recombinant adenovirus has been shown to inhibit the growth of gastric cancer cells with mutated p53 *in vitro* and *in vivo*[18,19]. P53 not only regulates cell cycle and cell growth but it also has a crucial role in activating proapoptotic genes such as Bax, Apaf-1, Fas and PTEN thus not only limiting cancer growth but also inducing apoptosis in cancer cells (if the proapoptotic genes are not mutated themselves). Other tumor suppressor genes useful in gastric cancer include: P16 gene and Fhit[20-23].

There are two approaches for inducing apoptosis in cancer cell lines, the first is to introduce native proapoptotic genes into cancer cells, this has the additional advantage of minimum toxicity of novel gene for non cancer cells (which already have a copy of native proapoptotic genes). The second approach on the other hand is to introduce genes of non-mammalian enzymes into cancer cells, so that they can convert non toxic prodrugs into highly toxic substances that will kill the cell[24]. This approach has the advantage of bystander effect, which means not only the infected cell will be killed but it will release the highly toxic drug which will kill the adjacent cells as well[25].

Among genes introduced into cells to induce apoptosis, Bax is a good example, as it independently of cell's P53 activity can make the cell commit suicide; it also has the further advantage of sensitizing the malignant cells to conventional anti-tumor treatments[26]. Some other genes that can be used in inducing apoptosis in gastric cancer cells include genes, which are effective in Caspase 3 common pathway including Caspase 8[27,28].

Another method of killing cancer cells is to transfect them with genes of non-mammalian enzymes which can convert non toxic prodrugs into highly toxic agents, destroying the infected cell and nearby cells. The most widely used suicide gene/prodrug system is the herpes simplex virus (HSV) thymidine kinase (HSV-tk)/ ganciclovir (GCV) system that can convert the prodrug GCV into phosphorylated GCV. The phosphorylated GCV inhibits cellular DNA synthesis and leads to the killing of cancer cells *via* apoptotic and non-apoptotic mechanisms[29,30]. A similar approach involves the expression of recombinant *E. coli* cytosine deaminase (CD) in gastric cancer cells together with the administration of 5-fluorocytosine (5-FC). 5-FC is given orally and converted to 5-fluorouracil in the tumor cells expressing CD[31] (Table 1).

Table 1 A review of recent methods used in gastric cancer adenoviral gene therapy

Apoptosis inducers & tumor suppressors	
Gene	**Conclusion**
FasL	Infection of human gastric carcinoma cells (SGC-7901) with Ad-FasL showed increased expression of FASL, resulting in apoptosis[32].
HDAC inhibitor	Induces apoptosis in cancer cells expressing wild and pseudo-wild type p53 *via* activating the p53 through acetylation[35].
E2F-1	E2F -1 is a transcription factor that regulates cell cycle progression into S-phase. Combining E2F-1 overexpression with cyclin-dependent kinase inhibitors results in an enhanced apoptotic response, causing nearly complete gastric tumor cell death[33].
Bax	In a study, Ad/Bax made marked Bax protein expression and effective apoptosis in MKN-1, MKN-7, MKN-28 gastric cell lines[34].
p51 and p53	p51 (p73L/p63/p40/KET), a p53 homologue, binds to p53-responsive elements to upregulate some p53 target genes and has been suggested to share partially overlapping functions with p53. p53 is an apoptosis promoting gene which could be used to eliminate tumor growth in gastric cancer. Adenovirally transduced p51A cDNA into human gastric cancer cells promotes apoptosis just like p53[36].
Caspase 8	Adv-Caspase 8 can selectively induce apoptosis in detached carcinoma cells[37].

ANTI ANGIOGENESIS GENES

Angiogenesis is essential for tumors in order to grow and form metastases. It is a multi step process, which is initiated by release of growth factors from cells, these growth factors include growth factors such as the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF)[38-40]. Inhibiting angiogenesis in tumors can in theory stop a tumor from functioning and growing, it is especially useful in limiting the dissemination of cancer[41-43]. Anti angiogenesis treatment do not need to specifically target cancer cells to inhibit metastasis as creating an anti angiogenesis environment around the cancer is equally effective. An example of anti angiogenesis genes is NK4, an HGF antagonist. It has been proved that transfecting gastric cancer cells with NK4 can stop the formation of Intra tumor vessels as well as peritoneal metastasis[44]. Another anti angiogenesis gene which can be introduced into cells using adenoviruses is soluble VEGF receptor (sFlt1) (Table 2).

TARGETED GENE THERAPY

One of the challenges of current gene therapy vector development, concerns targeting a therapeutic gene

Table 2 A review of recent methods used in gastric cancer adenoviral gene therapy

Metastasis inhibitors	
Gene	**Conclusion**
NK4	Peritoneal tumors in mice treated early with adenoviral mediated NK4 were significantly reduced. In another study Ad.CMV.NK4 resulted in efficient production and secretion of NK4 and significantly inhibited proliferation, migration and invasion of gastric cancer cells[45].
Flt-1	When administered intra peritoneally it markedly reduced the number of metastatic nodules larger than 1 mm in diameter on the peritoneal surface[46].
Caspase 8	In a study it was determined that Caspase 8 can augment anoikis in MKN-45 cells and suppress its peritoneal dissemination in nude mice with xenograft gastric cancer transplants and consequently increase survival, Caspase 8 has also proved to be useful in limiting metastasis in other carcinomas originating from epithelial tissues[37].

Table 3 A review of recent methods used in gastric cancer adenoviral gene therapy

Targeted gene therapy	
Method	**Conclusion**
HDAC inhibitor	Increase expression of the Coxackie adenovirus receptor and subsequent transfection efficiency of the adenovirus in cancer cells[35].
COX-2	COX-2 promoter shows the strongest cytocidal effect in gastric cancer cells when it is used in a conditionally replicating adenovirus (CRAD) context & with adenoviral vectors displaying 5/3 chimeric fibers[56].
EPCAM	It has been demonstrated that there is a marked difference in expression of the human epithelial cell adhesion molecule (EpCAM) between normal and (pre)malignant lesions of the stomach and esophagus. Based on this, using EpCAM to achieve gastric and esophageal adenocarcinoma selective gene transfer.may be a feasible choice for cancer-specific gene therapy[57]. In a study using EPCAM antibody adhered to adenovirus, transduction of normal gastric epithelium and liver tissue was reduced at least 10-fold in comparison with native adenovirus however tumor transduction levels remained the same[58].
Carcinoembryonic antigen (CEA)	A remarkable degree of targeted gene delivery to gastric cancer cells was obtained with Adv-FZ33 with the fully human anti-carcinoembryonic antigen (CEA) monoclonal antibody, C2-45[59-62].

to diseased cells with the aim of achieving sufficient gene expression in the affected tissue, while minimizing toxicity and expression in other tissues[47,48]. The use of recombinant adenoviruses as vectors for gene therapy is restricted by the widespread distribution of the coxsackie and adenovirus receptor (CAR), which allows infection of a range of tissues and precludes specific *in vivo* targeting[49-51].

Targeting can be achieved at the level of capsid binding or at later transduction events by the use of tissue-specific promoters. Targeting at the level of binding is preferred because even the interaction of cells with empty capsids leads to toxic effects. However a combination of both strategies has its obvious advantages.

Manipulating capsid binding can be achieved by direct genetic modifications of the capsid proteins, or it is possible to add ligands to the capsid *via* polymers like polyethylene glycol (PEG) and poly-[N-(2-hydroxypropyl) methacrylamide] (HPMA). Using these approaches, the native tropism of the virus is ablated either by the addition of polymer to fiber knob or the use of an anti fiber neutralizing antibody in the context of a bi-functional conjugate and creating targeted tropism by adding ligands. For example, for targeting ovarian cancer cells, it is possible to attach FGF to PEG bonded to viral capsid thus increasing specificity and also efficiency of transfection.

Targeting can also be achieved using tissue-specific promoters (TSP)[52-54], for example in alpha-fetoprotein (AFP)-producing gastric tumors, the adenovirus-mediated expression of HSV-tk by an AFP enhancer/promoter element selectively eliminates AFP positive, but not AFP-negative cell lines when treated with ganciclovir[55] (Table 3).

CONCLUSION

During the past 10 years, much has been learnt about molecular alterations in gastric cancer. Based on the understanding of the molecular mechanisms underlying gastric carcinogenesis, new therapeutic strategies targeted at the molecular defects in the tumor cells have been designed and many promising therapy results have been obtained from *in vitro* or *in vivo* studies. Among these approaches, Adenoviral gene therapy has proven to be the most promising however the efficiency of gene therapy for treatment of cancer in humans has remained low, this may be due to low rates of transduction and specificity. There may be many reasons for this, but it is widely agreed that this is mainly due to the relative resistance of cancer cells to introduce foreign material combined with low transgene expression *in vivo*. One of the most important issues affecting the possible clinical application of gene therapy is the need to ensure the highest possible safety levels developing protocols and targeting adenoviral vectors for gastric cell lines may yield the answer to this problem. Another prospect worth mentioning is the benefit of combining several approaches in order to achieve the highest possible therapeutic effect, examples of combined therapy approaches include combining Ad-p53 with HDAC inhibitor and sodium butyrate (SB), this has shown a significantly higher growth suppressive effect than single treatments of each. Another example shows combining NK4 with Cysplatin has enhanced inhibitory effect on peritoneal metastasis, suggesting that the combination of intra peritoneally chemotherapy with NK4 might be effective[63,64].

Recent studies have shed light on pathogenesis of gastric cancer, it has been long argued that *H Pylori* acts as a carcinogenic factor in inducing gastric adenocarcinoma, recent studies have supported this hypothesis, thus an area of future investigation in treatment of gastric cancer could involve *H Pylori* modification and also the intracellular

pathways altered by *H Pylori* in gastric mucosa. Although it has been reported that simple eradication of *H Pylori* without reversing the effects *H Pylori* has caused on gastric mucosa has limited efficacy in preventing gastric cancer, however there is a need for further investigation in this field[65-67].

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TOPIC HIGHLIGHT

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Non-alcoholic fatty liver disease and the metabolic syndrome: An update

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Abstract

Sedentary lifestyle and poor dietary choices are leading to a weight gain epidemic in westernized countries, subsequently increasing the risk for developing the metabolic syndrome and nonalcoholic fatty liver disease (NAFLD). NAFLD is estimated to affect approximate 30% of the general US population and is considered the hepatic manifestation of the metabolic syndrome. Recent findings linking the components of the metabolic syndrome with NAFLD and the progression to nonalcoholic steatohepatitis (NASH) will be reviewed; in particular, the role of visceral adipose tissue, insulin resistance, and adipocytokines in the exacerbation of these conditions. While no therapy has been proven effective for treating NAFLD/NASH, common recommendations will be discussed.

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Key words: Nonalcoholic fatty liver disease; Metabolic syndrome; Insulin resistance; Cytokines; Inflammation

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INTRODUCTION

Sedentary lifestyles and poor dietary choices are contributing to a weight gain epidemic in westernized societies. Recent epidemiological studies suggest an increased risk of cardiovascular disease (CVD) and type Ⅱ diabetes in overweight and obese individuals. Unfortunately, incidence of the metabolic syndrome and nonalcoholic fatty liver disease (NAFLD), which can precede the development of CVD and type Ⅱ diabetes, are also increasing.

The metabolic syndrome, a cluster of metabolic abnormalities with abdominal adiposity and insulin resistance as its central components, affects approximately 25% of the American adult population[1] and is associated with an increased risk of CVD and type 2 diabetes[2]. NAFLD, hepatic steatosis not due to alcohol consumption, is the most common cause of chronic liver disease[3]. It is estimated that about 30% of the general US population has excessive fat accumulation in the liver[3], reaching levels as high as 75%-100% in obese and morbidly obese individuals[4].

The relationship between NAFLD and the metabolic syndrome is becoming increasingly recognized. Approximately 90% of patients with NAFLD have ⩾ 1 characteristic feature of metabolic syndrome and about 33% have the complete diagnosis[5], placing NAFLD as the hepatic representation of the metabolic syndrome[6,7]. In addition, presence of the metabolic syndrome predicts higher risk for the development of NAFLD in both men and women[8]. NAFLD encompasses a histological spectrum ranging from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis, and cirrhosis. The majority of individuals with NAFLD have no symptoms with a normal physical examination; however, about 2%-6% of adult Americans and 20% of those who are obese may develop steatosis with inflammation (NASH), fibrosis, and cirrhosis[9]. Furthermore, there appears to be a close link between the metabolic syndrome, low grade inflammation, and oxidative stress[10].

In this editorial, we will review the clinical links between the components of the metabolic syndrome with

the manifestations of NAFLD and the progression to nonalcoholic steatohepatitis (NASH). In addition, we will discuss the potential role of adipocytokines and oxidative stress in the exacerbation of these conditions. Further, we will briefly discuss commonly prescribed therapeutic treatments.

DIAGNOSIS AND PREVALENCE OF THE METABOLIC SYNDROME

Although an association between different metabolic abnormalities had been noted for several years, the metabolic syndrome was first publicly described in 1988 by Reaven[11]. Then called Syndrome X, the metabolic syndrome consisted of a cluster of metabolic abnormalities, including obesity (especially abdominal obesity), insulin resistance, impaired glucose metabolism, dyslipidemia, and elevated blood pressure[11]. The current definition of the metabolic syndrome varies depending on the position of different regulating bodies[12], and it is not within the scope of this review to delineate the best available definition.

The metabolic syndrome is estimated to affect about 25% of the American adult population[1], and the prevalence will continue to increase dramatically. Regardless of the precise definition, the hallmark features of the metabolic syndrome are impaired glucose tolerance/insulin resistance, hypertension, central adiposity, and dyslipidemia consisting of low high-density lipoprotein-cholesterol (HDL-C) and elevated plasma triglycerides (TG). It is largely believed that insulin resistance is the central feature in the development of the metabolic syndrome.

DIAGNOSIS AND PREVALENCE OF NAFLD

It is estimated that about 30% of the US adult population has NAFLD, approximately 16%-20% of non-obese individuals and as high as 76% and 100% prevalence in the obese and morbidly obese individuals, respectively[4]. Overall, NAFLD is estimated to affect over 90 million Americans. NAFLD is characterized by increased accumulation of triglycerides (TG) in hepatocytes (hepatic steatosis). The diagnosis of NAFLD requires that hepatic steatosis be $\geqslant$ 5% by weight in the absence of excess alcohol consumption (> 20 g/d)[13]. Most people with NAFLD, especially those with fatty liver but no inflammation, experience little to no problems from the condition. The diagnosis usually is first suspected in an overweight or obese person who is found to have mild elevations in specific liver enzymes measured in circulation including elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST), with ALT levels being greater than AST levels.

Currently, the liver biopsy is considered the "gold standard" for the direct measurement of hepatic fat and is the only reliable method for diagnosing fatty liver and/or NASH. However, the liver biopsy has limited applicability in epidemiological and clinical studies and is not routinely performed because of the invasiveness of the procedure. Several non-invasive methods are currently available, which include ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS). Each has its limitations and is only semi-quantitative, but is more easily employed for routine screening.

LINKING THE METABOLIC SYNDROME AND NAFLD

Hyperinsulinemia and insulin resistance play a major role in the pathogenesis of NAFLD[14]. Because insulin resistance is the key component of the metabolic syndrome (often referred to as the insulin resistance syndrome or pre-diabetes) and is also commonly found with obesity, and because these same factors are commonly associated with NAFLD, there is a plethora of accumulating evidence supporting an association between obesity, the metabolic syndrome, and NAFLD[6,7]. In fact, NAFLD is now considered to be the hepatic representation of the metabolic syndrome[14,15].

The metabolic syndrome, in part through glucose intolerance and insulin resistance, is strongly associated with steatosis, fibrosis, and cirrhosis of the liver in severely obese adults[16]. In addition, central fat distribution, fatty liver, and glucose intolerance are noted in mildly obese and in normal weight subjects[17,18]. Further, numerous studies have demonstrated that obesity, type 2 diabetes, dyslipidemia, hypertension, and insulin resistance are strongly associated with NAFLD[12,19-22]. NAFLD also is strongly associated with hepatic, adipose tissue, and whole body reductions in insulin sensitivity, increased rate of gluconeogenesis, impaired insulin response to suppress gluconeogenesis, and impaired fatty acid oxidation[23-25]. However, the question about whether hepatic insulin resistance is a cause or a consequence of hepatic steatosis is unresolved[26-28].

The metabolic syndrome predicts higher risk of NAFLD in men and women, independent of weight gain[8], and those individuals with the metabolic syndrome are less likely to experience regression of NAFLD[8]. In addition to insulin resistance, body mass index and waist circumference, indices of obesity and central adiposity, also are associated with the metabolic syndrome, insulin resistance, fibrosis, and steatohepatitis[29], and both liver fat and visceral adipose tissue stores are associated with metabolic risk[30]. Moreover, HDL-C concentrations are significantly reduced[24] and serum TG and cholesterol levels are elevated with NAFLD[24,30]. Collectively, it appears that insulin resistance is not the only contributing factor in the development of NAFLD, but rather, a combination of multiple stimuli.

IS THE METABOLIC SYNDROME MEDIATING THE PROGRESSION OF NAFLD?

Physical inactivity and poor nutritional choices are leading to a surge in obesity, insulin resistance, and metabolic syndrome in industrialized nations. Day and James[31] proposed the "two-hit hypothesis" to explain the presence of bland steatosis and the progression to inflammation

(NASH), fibrosis, and cirrhosis. Recent findings suggest that components of the metabolic syndrome are integrally involved in the pathogenesis of this two hit model.

The first "hit" is hepatic steatosis, NAFLD in its simplest form, develops from an imbalance in triglyceride formation *vs* turnover. We and others have shown that mitochondrial dysfunction (i.e. impaired mitochondrial β-oxidation) is a primary cause of hepatic steatosis (discussed in detail by Wei *et al* in this issue)[23,32,33]. Insulin resistance is a near essential requirement and is believed to influence the first "hit" of NAFLD in the following ways: (1) activate the secretion of harmful adipocytokines from adipocytes, (2) alter rates of hepatocyte TG synthesis and transport, (3) and finally increases lipolysis [hydrolysis of TG to free fatty acids (FFA)] rates in central adipose which are dumped into the portal vein where they are processed in the liver, exposing the liver to excessive FFA levels[12]. In addition to impaired mitochondrial function and conditions exacerbated by insulin resistance, elevated hepatic *de novo* lipogenesis and TG synthesis strongly contributes to hepatic steatosis. In fact, a recent study found that about 26% of hepatic TG accumulation in NAFLD patients could be accounted for by *de novo* lipogenesis[34].

Once hepatic steatosis is present, other factors, such as inflammation and oxidative stress, are thought to promote progression to NASH, fibrosis, and necrosis. The metabolic syndrome is linked to inflammation and oxidative stress[10], and it has been demonstrated that individuals with the metabolic syndrome have increased lipid peroxidation[35]. The second "hit" likely is comprised of a secondary insult, particularly by adipocytokines and reactive oxygen species, which activate stellate cells and increase fibrogenesis and lipid peroxidation. Hepatic inflammation and fibrosis are associated with the presence and severity of the metabolic syndrome[36]. Furthermore, the presence of steatohepatitis with fibrosis, confirmed with liver biopsy, is associated with increased waist circumference and body mass index[29]. Hyperglycemia and hypertriglyceridemia also are associated with NASH[37], and insulin resistance is more severe in individuals with NASH *vs* simple fatty liver[37]. If the insults are great enough and the patient develops cirrhosis, about 33% of these patients will die or develop morbid conditions[38].

THE METABOLIC SYNDROME AND COMMON LIVER MARKERS

Circulating concentrations of the liver transaminases, ALT, AST, and to less extent γ-glutamyltransferase (GGT) are commonly used as markers of NAFLD for many years. Elevated levels are considered a consequence of liver damage due to fatty acid infiltration and inflammatory stimuli, and recent findings indicate that serum levels of these enzymes are associated with multiple components of the metabolic syndrome.

Increases in ALT are positively associated with each component of the metabolic syndrome, increased TG, glucose, waist circumference, diastolic blood pressure, and reduced HDL-C levels[19]. In addition, insulin resistance, the prevalence of severe liver steatosis, and ALT values have been found to be significantly higher in subjects with the metabolic syndrome compared to those with less than three of the five clinical features considered for its diagnosis[39]. Recent data published from NHANES Ⅲ found significant association with increased ALT and insulin resistance, type Ⅱ diabetes, and the metabolic syndrome[40]. GGT, a less specific marker of liver function, is linked to obesity, hypertension, sedentary lifestyle, hyperinsulinemia, dyslipidemia, inflammation, and oxidative stress[41-45]. Furthermore, GGT concentrations have been found to be associated with hypertension in individuals with central adiposity[46], suggesting the potential for a pathogenic link among fatty liver disease, endothelial dysfunction, and cardiovascular risk.

ROLE OF ADIPOSE TISSUE, FREE FATTY ACIDS, AND ADIPOCYTOKINES

Adipose tissue

Adipose tissue is now recognized as not simply a storage depot for excess energy, but rather, an active endocrine organ that secretes a number of molecules termed, adipocytokines. A number of these adipocytokines have been linked to alterations in insulin sensitivity, including adiponectin, leptin, resistin, and tumor necrosis factor-α (TNFα). In addition, many of the same signaling molecules have been shown to be associated with suppressed hepatic insulin sensitivity[47,48], and it is thought that adipocytokines may contribute to the development of liver fibrosis.

Visceral adipose tissue is more biologically active than subcutaneous adipose tissue and is known to release greater quantities of adipocytokines. Visceral adipose tissue is a better predictor of altered liver function and insulin resistance than obesity defined by body mass index[49,50]. FFAs lipolyzed from visceral adipocytes are dumped directly into the portal vein where they circulate through the liver and with suppressed oxidative capacity seen in insulin resistance will overload hepatocytes with lipid and promote hepatic TG storage.

Free fatty acids

The liver represents a major site of glucose uptake and storage and the major site for insulin clearance. Under normal insulin sensitive conditions, insulin inhibits glycogenolysis and gluconeogenesis by the liver, suppressing glucose production. However, both the metabolic syndrome and NAFLD commonly lead to hepatic insulin resistance in which the most common feature is the inability of insulin to cease hepatic glucose production. Boden *et al*[51] demonstrated that hepatic insulin resistance could be induced in rats by infusing lipids during a euglycemic-hyperinsulinemic clamp, in part by elevating hepatic diacylglycerol concentrations and increasing the activation of protein kinase C, IKκB, and NF-κB. Moreover, free fatty acids (FFA) and lipid intermediate flux from adipocyte lipolysis or from excess dietary intake activate protein kinase C and phosphorylate serine residues on the insulin receptor and insulin receptor

substrates, impairing tyrosine phosphorylation and increasing hepatic insulin resistance which likely lead to elevated glucose production and persistent hyperglycemia.

The liver also responds to elevated FFA by increasing cholesteryl ester synthesis, production of very low density lipoproteins[52], and *de novo* TG synthesis, exacerbating dyslipidemia. In addition, elevated FFA also have toxic effects on the liver due to lipid peroxidation, resulting in fibrogenesis and progression to cirrhosis[53]. Furthermore, lipid-laden hepatocytes also can swell and this architectural distortion can influence hepatocyte health[54].

Leptin

The role of leptin and leptin resistance in NAFLD has been under recent investigation. Under normal, healthy conditions, leptin is thought to play a role in regulation of body weight. However, in human obesity, leptin concentrations are elevated but there is reduced responsiveness to available leptin presumably from reduced leptin receptor expression, resulting in leptin resistance either centrally or locally at the level of the liver. Leptin is thought to play a major role in hepatic TG accumulation[55], and hepatic steatosis is observed in ob/ob and db/db mice that have leptin and leptin receptor mutations, respectively. Elevated leptin levels are associated with increased serum ALT levels and could be involved in promoting hepatocellular injury[56]. However, leptin therapy has been shown to reverse insulin resistance and hepatic steatosis in individuals with lipodystrophy[57]. In addition, existing data from animal studies also support the role of leptin replacement in reducing TG synthesis and inducing β-oxidation[58]. Future studies should focus on leptin receptor expression and the bioavailability of leptin and associations with NAFLD and the metabolic syndrome.

Resistin

Resistin has recently been identified as a signaling molecule that is induced during adipogenesis and secreted by adipocytes, particularly visceral adipose tissue stores[48,59]. Resistin was identified on the basis of an adipocyte specific protein that played a role in insulin resistance. Little attention has been given to the role of resistin in the metabolic syndrome and NAFLD, but available findings targeting resistin appear promising. Resistin levels are increased in NAFLD patients and levels are related to the histological severity of the disease[60]. Resistin gene expression and protein secretion are markedly reduced by anti-diabetic thiazolidinediones (TZDs), and administration of anti-resistin antibody has been shown to improve blood glucose and insulin action in mice with diet-induced obesity[59]. In addition, weight loss interventions in humans have demonstrated significant reductions in circulating concentrations of resistin[48].

TNFα

Adipose tissue represents a site for significant macrophage accumulation, the major source of local TNFα expression[61]. TNFα is an adipocytokine with a well-known role in antagonizing the effects of adiponectin and contributing to insulin resistance[62]; however, recent observations suggest that it also is involved in the metabolic syndrome and progression of NAFLD. In particular, TNFα has been shown to play an important role in mouse models of obesity and insulin resistance, and there also appears to be a link between TNFα levels and liver mitochondrial dysfunction that contributes to NAFLD and NASH. TNFα is elevated in individuals with central adiposity and other components of the metabolic syndrome[63]. It is thought that overproduction of TNFα in liver plays a pathogenic role in NAFLD, as TNF mRNA and TNF receptor 1 mRNA levels are increased in patients with NASH[64]. Furthermore, TNFα alters apolipoprotein metabolism by suppressing apoE secretion and apoA1 expression in HepG2 cells[65] and rapidly down-regulates the anti-oxidative protein associated with high-density lipoprotein, paraxonase-1 (PON1)[66].

Adiponectin

Adiponectin is an adipocyte-specific secretory protein that is found in relatively high circulating levels (5-10 mg/L), but decreased concentrations predict the incidence of CVD, the metabolic syndrome, and NAFLD[67-71]. Adiponectin concentrations are decreased in patients with obesity, insulin resistance, type 2 diabetes, and NAFLD[69,72] and correlate negatively with hepatic fat content[28]. In addition, hyperinsulinemia down regulates adiponectin receptor expression[73], reducing overall biological activity. Adiponectin has antilipogenic effects that may protect non-adipocyte tissues like liver and muscle. Adiponectin stimulates mitochondrial β-oxidation by activating AMP-dependent protein kinase (AMPK)[74] and down regulates a key transcription factor in *de novo* fatty acid synthesis, sterol regulatory element binding protein 1-c (SREBP-1c)[75]. This, in turn, reduces malonyl-CoA levels and the inhibition on carnitine palmityl transferase-1, leading to increased fatty acid oxidation and reduced hepatic TG accumulation[76].

Adiponectin also displays anti-oxidative/inflammatory properties as it may antagonize the effects of inflammatory mediators like TNFα and attenuate the progression of NAFLD by reducing hepatic stellate cell proliferation and increases apoptosis[77]. Furthermore, administrations of recombinant adiponectin in ob/ob mice, a model of NASH, attenuates serum ALT levels and drastically reduces hepatic steatosis[78].

Regardless whether adipocytokines are merely markers of the presence of NAFLD or are playing pivotal roles in the pathogenesis of the disease, existing evidence supports further study of adipocytokines as possible therapeutics for human NAFLD. It appears that alterations in adipocytokines secretion are exacerbating insulin resistance in skeletal muscle and liver and promoting hepatic steatosis and the development of NASH.

THERAPIES OF NAFLD AND THE METABOLIC SYNDROME

With no proven treatment for NAFLD/NASH, the focus of previous investigations has been on the treatment of components of the metabolic syndrome (obesity, hypertension, dyslipidemia, and diabetes). This strategy has

been met with some success and available therapies appear to slow NAFLD development and progression.

Pharmacological interventions

The strong relationship between insulin resistance and NAFLD suggests that insulin sensitizing therapies (TZDs and metformin) might be beneficial in the prevention or improvement in NAFLD. TZDs and metformin are oral glucose-lowering medications used to treat type 2 diabetes that enhance insulin sensitivity and signaling. TZDs bind to the peroxisome proliferator-activated receptors (PPARs) and improve insulin sensitivity, in part, by facilitating enhanced TG storage by adipocytes, suppressing the ectopic storage of lipids into liver and skeletal muscle. In addition, TZDs appear to have anti-inflammatory properties, inhibiting adipocyte gene expression and reduce circulating levels of TNFα and resistin[79], and increase adiponectin concentrations[80]. However, it should be noted that a common side effect of TZDs in clinical trials is weight gain.

Metformin is the drug of choice for treating obesity and type Ⅱ diabetes, and additional findings support its use for treating NAFLD, although the precise mechanism(s) of action are not known. It is known, however, that metformin targets and activates AMPK[81], which has multiple beneficial effects. Administration of metformin reduces hepatic gluconeogenesis, decreases absorption of glucose from the gastrointestinal tract, and increases insulin sensitivity. It also has been shown to reduce hepatic lipogenesis, increase fatty acid oxidation, and reduce serum ALT levels, steatosis, and inflammation in the ob/ob mouse model of NAFLD[82]. Furthermore, metformin use in non-diabetic NAFLD patients has been shown to reduce liver fat by 50% and decrease liver inflammation and necrosis[83].

Lipid lowering agents also can lower risks of the metabolic syndrome and NAFLD. It is well known that statins combat dyslipidemia, a hallmark of the metabolic syndrome, by reducing serum TG and increasing HDL-C levels; however, statins also have been shown recently to improve liver enzymes and hepatic inflammation (reviewed in[84]). Fibrate administration also has been met with marginal success. Furthermore, the angiotensin system is known to play a role in hypertension, and it is thought to be involved in the pathogenesis of hepatic fibrosis and insulin resistance. Although only preliminary evidence exists, treatment with the angiotensin Ⅱ receptor antagonist losartan beneficially improved blood markers of hepatic fibrosis and serum ALT levels[85]. Large, randomized control trials are lacking, but future studies are warranted.

Lifestyle modifications

Lifestyle modifications targeted at increasing physical activity and reducing energy intake are recommended by health care providers for optimal health and are the most common prescribed therapy for individuals diagnosed with NAFLD. In addition, recent cross-sectional studies in humans provide evidence that increased habitual physical activity[86] and cardiorespiratory fitness[87] are inversely associated with NAFLD. However, there are relatively few prospective studies that have examined the effects of aerobic training on the development and prevention of hepatic steatosis in humans or animal models.

Managing body weight is likely the best overall method in the treatment of conditions in both the metabolic syndrome and NAFLD. Since insulin resistance and obesity play a central role in NAFLD, weight loss is the mainstay of treatment. The weight loss program should be centered on the caloric restriction rather than alteration of dietary contents, with a prescription of both aerobic and resistance training exercise. Initial goals from the patient should be a 10% reduction in body weight by the combination of exercise training and energy restriction. This should be targeted over a 6 mo period, and it is important that individuals continue exercising to maintain the lost weight and for overall cardiometabolic health.

Weight loss by energy restriction significantly reduces hepatic TG content, rates of endogenous glucose production, and increases insulin suppression of endogenous glucose production in type 2 diabetics[88]. Surgical weight loss has been reported to reduce prevalence of the metabolic syndrome, liver steatosis, inflammation, and fibrosis[89]. However, these findings are not universal and it has been suggested that rapid weight loss may in fact, significantly increase hepatic steatosis, presumably from increased FFA flux from elevated rates of lipolysis. Exercise training in combination with energy restriction significantly reduces fatty liver and serum ALT concentrations in obese individuals with NAFLD[90]. Furthermore, weight loss (10%) by diet and exercise significantly elevates adiponectin concentrations[72]. Moreover, we recently have shown that weight loss by diet and exercise significantly reduces circulating leptin concentrations[91] and improves insulin sensitivity and lipid peroxidation in overweight and obese adults with components of the metabolic syndrome[92].

CONCLUSION

Most experts consider NAFLD to be the hepatic manifestation of the metabolic syndrome. The progression from hepatic steatosis to steatohepatitis may worsen insulin resistance and dyslipidemia, or the worsening of peripheral factors may mediate alterations in the liver. Regardless of the initiating pathophysiological event(s), recent findings emphasize the important links between NAFLD and the metabolic syndrome. Future investigations targeting the components of the metabolic syndrome as they exacerbate conditions of NAFLD are warranted. Furthermore, weight loss and insulin-modulating pharmacologic agents are the most effective treatment thus far, but combination therapies of lifestyle modifications and pharmacological agents could prove to be more effective than the individual prescription of specific drugs.

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TOPIC HIGHLIGHT

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Nonalcoholic fatty liver disease and mitochondrial dysfunction

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Abstract

Nonalcoholic fatty liver disease (NAFLD) includes hepatic steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. NAFLD is the most common liver disorder in the United States and worldwide. Due to the rapid rise of the metabolic syndrome, the prevalence of NAFLD has recently dramatically increased and will continue to increase. NAFLD has also the potential to progress to hepatocellular carcinoma (HCC) or liver failure. NAFLD is strongly linked to caloric overconsumption, physical inactivity, insulin resistance and genetic factors. Although significant progress in understanding the pathogenesis of NAFLD has been achieved in years, the primary metabolic abnormalities leading to lipid accumulation within hepatocytes has remained poorly understood. Mitochondria are critical metabolic organelles serving as "cellular power plants". Accumulating evidence indicate that hepatic mitochondrial dysfunction is crucial to the pathogenesis of NAFLD. This review is focused on the significant role of mitochondria in the development of NAFLD.

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Key words: Nonalcoholic fatty liver disease; Mitochondria; Fatty acid oxidation; Liver

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) encompasses a disease spectrum ranging from simple hepatic steatosis to steatohepatitis (NASH), fibrosis, and cirrhosis. NAFLD has a very high prevalence in the US and worldwide[1]. It is becoming the leading cause for referral to liver clinics in most areas. In the US, it occurs in about 20%-35% of the population in over 60 million of the general adult population. Further, NAFLD occurs in about 2.6% of children and up to 53% of obese children are diagnosed with NAFLD[2,3]. The prevalence of NAFLD will likely continue to rise. Obesity, hyperglycemia, type 2 diabetes and hypertriglyceridemia are most important risk factors. Genetic factors undoubtedly predispose to NAFLD, as supported by higher prevalence of steatosis in Hispanics than Caucasians and African-Americans[4]. NAFLD has the potential to progress to hepatocellular carcinoma (HCC) or liver failure, both events that ultimately lead to early death.

DEFINITION AND CHARACTERISTICS OF NAFLD

NAFLD is defined as an excess of fat in the liver in which at least 5% of hepatocytes display lipid droplets[5] that exceed 5%-10% of liver weight[6,7] in patients who do not consume significant amounts of alcohol (140 g ethanol/week for men and 70 g ethanol/week) for women[8]. However, this definition is still controversial, because there is only 1.7% ± 0.2% of liver fat content in some healthy men[9] and liver fatty accumulation is absent in hepatic cirrhosis[10]. Morphologically, hepatic steatosis manifests as accumulation of macrovesicular or microvesicular intracytoplasmic fat droplets in hepatocytes. In macrovesicular steatosis, a large single fat vacuole fills the cytoplasm of hepatocytes and displaces the nucleus to the periphery, causing a characteristic signet ring appearance. In microvesicular steatosis, numerous small lipid droplets occupy the cytoplasm of hepatocytes and do not displace the nucleus to the periphery. Hepatic steatosis can be reversible or progress to NASH depending on the cessation or persistence of the underlying provocative cause, respectively. NASH represents steatosis, inflammation, fibrosis, ballooning hepatocytes, apoptotic cells and Mallory's hyaline. The inflammatory extent varies considerably and does not always correlate with the degree of steatosis. Infiltrating inflammatory

cells consist of lymphocytes and polymorphonuclear leukocytes. Apoptotic ballooned and Mallory hyaline hepatocytes may indicate the onset of NASH. Liver cell death and inflammation activate stellate cells leading to the development of hepatic fibrosis that often commences in zone 3 and manifests as perisinusoidal, perivenular (around terminal hepatic veins), and pericellular fibrosis. Hepatic steatosis, inflammation and aggressive fibrogenesis as well as sustained hepatocellular proliferation contribute to the development of liver cirrhosis.

DIAGNOSIS OF NAFLD

The majority of patients with NAFLD are asymptomatic or may complain of fatigue, exercise intolerance, or unspecific, vague abdominal pain in the right upper quadrant[5]. Physical examination can be unremarkable, although a palpable enlarged liver is frequent. Liver tests usually show minor nonspecific abnormalities. Alanine aminotransferases and gamma-glutamyl transpeptidase levels are elevated in most cases, but may be normal in some patients with advanced NASH and hepatic cirrhosis[11]. Imaging techniques including ultrasonography and magnetic resonance imaging are useful to detect degrees of steatosis, but can not distinguish inflammatory activity and fibrosis. Liver biopsy is still the gold standard for the diagnosis of NASH and fibrotic severity[12-14] which can be used to evaluate the degree of steatosis, inflammation, and fibrosis and also can help exclude other causes of liver disease[15].

PATHOGENESIS OF NAFLD

A currently favored hypothesis is that "two hits" are required for a subject to develop NASH[16]. The first hit leads to hepatic steatosis, and the second to hepatocyte injury and inflammation. However, the primary metabolic abnormalities leading to lipid accumulation within hepatocytes has remained poorly understood. Accumulating evidence indicates that mitochondrial dysfunction plays a central role in the pathogenesis of NAFLD, and that NAFLD is a mitochondrial disease[17].

Liver and metabolism

The liver, as a super metabolic achiever in the body, plays a critical role in metabolism of carbohydrate, protein and fat to maintain blood glucose and energy homeostasis.

Hepatic mitochondria: Mitochondria serve as the cellular powerhouse that generates ATP or heat by using substrates derived from fat and glucose. Hepatocytes are normally rich in mitochondria and each hepatocyte contains about 800 mitochondria occupying about 18% of the entire liver cell volume. Mitochondria play an important role in hepatocyte metabolism, being the primary site for the oxidation of fatty acids and oxidative phosphorylation. A mitochondrion contains inner and outer membranes composed of phospholipid bilayers and proteins. The outer mitochondrial membrane contains numerous integral proteins called porins, which contain a relatively large internal channel that is permeable to all molecules of 5000 daltons or less. Larger molecules can only traverse the outer membrane by active transport through mitochondrial membrane transport proteins. Unlike the outer membrane, the inner membrane does not contain porins, and is highly impermeable; almost all ions and molecules require special membrane transporters to enter or exit the matrix. The inner mitochondrial membrane contains proteins with four types of functions: the oxidation reactions of the respiratory chain; ATP synthase; specific transport proteins that regulate the passage of metabolites into and out of the matrix; protein import machinery. The matrix contains a highly concentrated mixture of hundreds of enzymes. Of the enzymes, the major functions include oxidation of pyruvate and fatty acids, and the citric acid cycle. The mitochondrial respiratory chain (MRC) is extremely important for energy generation and it consists of multiple polypeptides. Most of the respiratory chain polypeptides are encoded by nuclear DNA, but some are encoded by mitochondrial DNA (mtDNA). mtDNA is a circular double-stranded molecule located in the mitochondrial matrix. mtDNA is extremely sensitive to oxidative damage due to its proximity to the inner membrane, the absence of protective histones, and the incomplete DNA repair mechanisms in mitochondria. Therefore, any factors that affect mitochondrial integrate will cause reduced mitochondrial function.

Hepatic free fatty acids sources: (a) dietary triglycerides (TG) as chylomicron particles from the intestine; (b) *de novo* synthesis in the liver; (c) Free fatty acids (FFA). influx into the liver from lipolysis of adipose tissue; (d) diminished export of lipids from the liver; and (e) reduced oxidation of fatty acids. Imbalance of these metabolic steps will increased TG accumulation within the cytoplasm of hepatocytes.

Hepatic mitochondrial fatty acid oxidation: Fatty acid oxidation (FAO) is the major source of energy for skeletal muscle and the heart, while the liver oxidizes fatty acids primarily under the conditions of prolonged fasting, during illness, and during periods of increased physical activity. FAO also plays an essential role in the intermediary metabolism of the liver. The oxidation of fatty acids in the liver fuels the synthesis of ketone bodies, 3-hydroxy butyrate and acetoacetate, which are utilized as alternative sources of energy by extrahepatic organs, like the brain when blood glucose levels are low. FAO occurs in three subcellular organelles, β-oxidation in mitochondria and peroxisomes, and ω-oxidation in the endoplasmic reticulum[18,19]. Mitochondrial β-oxidation is the dominant oxidative pathway for the disposal of fatty acids under normal physiologic conditions and is primarily involved in the oxidation of short-chain ($< C_8$), medium-chain (C_8-C_{12}), and long-chain (C_{12}-C_{20}) fatty acids. Short-chain and medium-chain FFAs freely enter the mitochondria, while long-chain FFAs entry into the mitochondria is regulated by the activity of the enzyme carnitine palmitoyl transferase I (CPT- I). Glycolysis generates pyruvate, which is transformed by mitochondria into acetyl-CoA, an intermediate that goes through the citric acid cycle for the production of reducing agents and ATP. However, when glucose and energy levels are

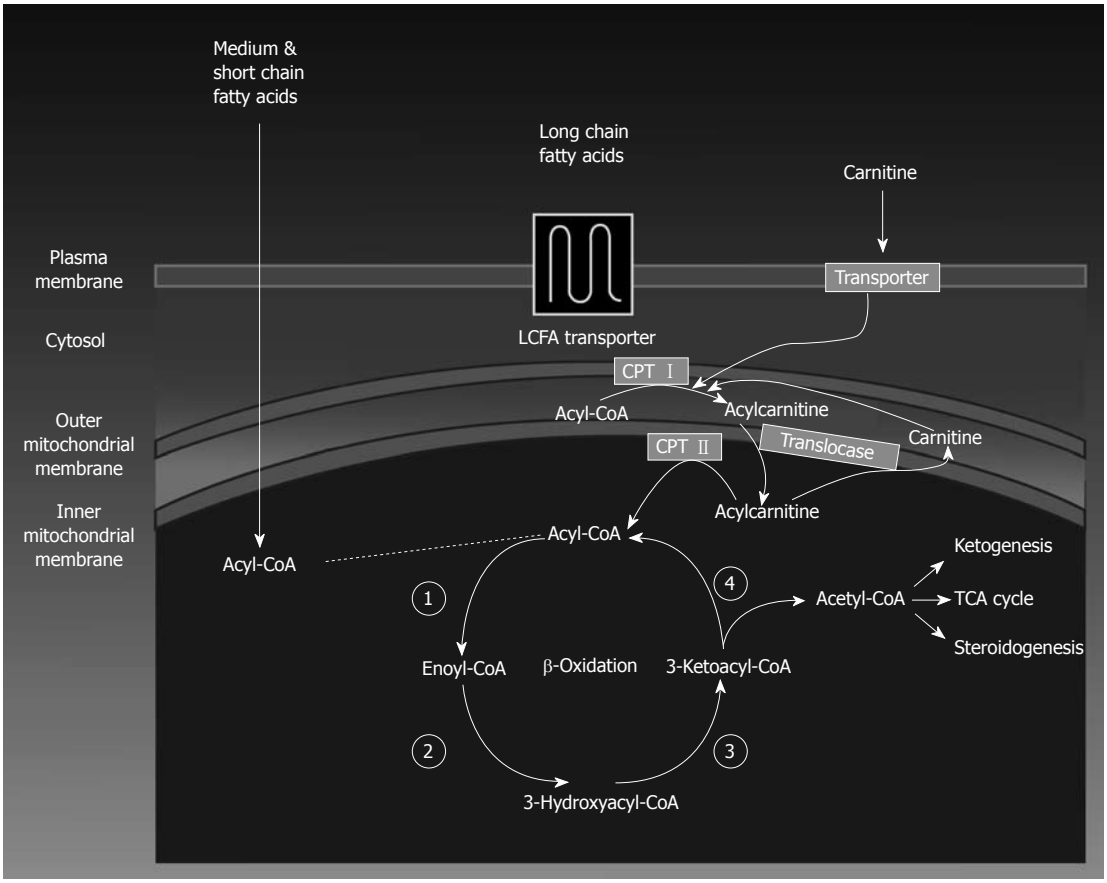


Figure 1 An illustration of mitochondrial fatty acid β-oxidation. LCFA: long-chain fatty acid; TCA: tricarboxylic acid.

elevated, acetyl-CoA is converted to citrate which can leak out of the mitochondrial matrix into the cytosol through the tricarboxylate carrier. In the cytosol, citrate regenerates acetyl-CoA which is converted to malonyl-CoA by acetyl-CoA carboxylase. Malonyl-CoA plays important roles in both hepatic fatty acid oxidation and lipid synthesis. Malonyl-CoA is the initial component for fatty acid synthesis. High malonyl-CoA levels also actively inhibit CPT- I enzyme activity thus robustly decreasing fatty acid oxidation by reducing the rate of fatty acid entry into the mitochondria. Thus, periods of caloric overconsumption, and excessive energy supply, increase malonyl-CoA levels which promotes hepatic fatty acid synthesis (storage) and suppresses fatty acid oxidation (catabolism). Conversely, in the fasting state, hepatic malonyl-CoA levels are low, allowing extensive mitochondrial import of long-chain FFAs and high rates of β-oxidation. Figure 1 depicts the pathway for mitochondrial fatty acid oxidation. During β-oxidation in mitochondria, FFAs undergo a dehydrogenation, then hydration, followed by a second dehydrogenation, and finally thiolysis, releasing one 2-carbon acetyl-CoA molecule and a shortened fatty acid (Figure 1). The cycle is repeated to split the fatty acid into acetyl-CoA subunits. The acetyl-CoA units enter the citric acid cycle to produce reducing agents which can be converted to ATP in the electron transport chain. Under fasting conditions, acetyl-CoA moieties can be converted into ketone bodies (acetoacetate and β-hydroxybutyrate), which are released by the liver to be oxidized in peripheral tissues by the tricarboxylic acid cycle.

MITOCHONDRIAL DYSFUNCTION IN NAFLD

Although the mechanisms responsible for fatty liver are still not fully elucidated, decreased capacity to oxidize fatty acids, increased delivery and transport of FFAs into the liver, and augmented hepatic fatty acid synthesis are likely to play a significant role in the pathogenesis of NAFLD. We and others have shown that mitochondrial abnormalities are closely related to the pathogenesis of NAFLD which raised the possibility that NAFLD is a mitochondrial disease[17,20-22]. The mitochondrial abnormalities associated with NAFLD include ultrastructural lesions, depletion of mitochondrial DNA (mtDNA), decreased activity of respiratory chain complexes, and impaired mitochondrial β-oxidation. Abnormal morphologic changes in liver mitochondria have been observed in patients and animal models with NASH[18,20-24]. Electronic microscopy revealed that mitochondria in NAFLD are big and swelled, scarce in number, and that the mitochondrial matrix has paracrystalline inclusions and hypodensity. Figure 2 shows an example of the ultrastructural changes in the mitochondrial in a mouse model with a fatty acid oxidation defect and hepatic steatosis that we have generated and reported earlier[22]. We have also found similar mitochondrial lesions in other rodent models that develop hepatic steatosis/NASH (data not shown). These same mitochondrial lesions are found in liver biopsy specimens from patients treated with 4,4'-diethylaminoethoxyhe xestrol, a drug that inhibits mitochondrial respiratory

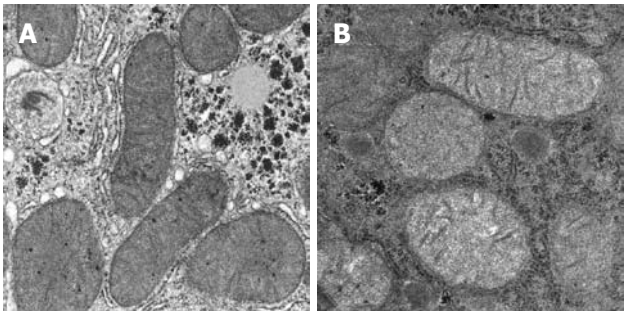


Figure 2 Representative electron micrograph of hepatocytes from control wild-type mice (MTPa$^{+/+}$) (**A**) and mice heterozygous for a mitochondrial trifunctional protein defect (MTPa$^{+/-}$) (**B**) at 11 700 × magnification. The MTPa$^{+/-}$ mice develop hepatic steatosis (see Figure 3). The mitochondria from the MTPa$^{+/-}$ mice were swollen with hypodense matrix and disrupted cristae. Reproduced with permission from *Gastroenterology*. 2005; 128: 1381-1390.

chain (MRC) activity and mitochondrial β-oxidation[25]. Prolonged treatment with this agent is associated with hepatic steatosis and steatohepatitis that is histologically indistinguishable from NAFLD in humans[25]. The ultrastructural mitochondrial defects in patients with NAFLD may be indicative of defective mitochondrial functions, e.g. reduced MRC activity[26] and impaired ATP synthesis[27]. NAFLD is often found in patients with insulin resistance, obesity and type 2 diabetes, the same metabolic conditions in which there is decreased oxygen consumption and ATP production, reduced total mtDNA and mtDNA transcription factor A, and reduced content of respiratory proteins in the fat, muscle and liver[28]. Many genes encoding mitochondrial proteins in skeletal muscle and fat are negatively correlated with body mass[29-33]. mtDNA depletion in hepatocytes impairs mitochondrial function and causes hepatic steatosis and other liver injury[34,35]. Patients with NASH have decreased expression of mtDNA-encoded polypeptides[36] and low activity of complexes Ⅰ, Ⅲ, Ⅳ and Ⅴ[26]. Mice with genetic deletion of NEIL1 DNA glycosylate have increased mtDNA damage and deletions and develop fatty liver disease[37].

Multiple enzymes are involved in mitochondrial β-oxidation and deficiency of these enzymes may lead to the development of hepatic steatosis, e.g. mice with disrupted medium-chain and very-long-chain acyl-CoA dehydrogenase genes manifest defects in fatty acid oxidation that likely lead to the witnessed micro- and macrovascular hepatic steatosis found in these mice. Mitochondrial trifunctional protein (MTP) is a heterotrimeric protein that consists of four α-subunits and four β-subunits and catalyzes long-chain fatty acid oxidation. MTP defects in humans are recessively inherited, and children with defects of any of the three enzymatic activities exhibit mostly microvesicular hepatic steatosis. We have generated a mouse model for a null mutation causing complete MTP deficiency and demonstrated that homozygous mice develop hepatic steatosis immediately after birth[38]. In subsequent report, we have documented that aging mice heterozygous for the MTP defect also become insulin resistant and develop hepatic steatosis as shown in Figure 3[22]. Further, the activity of mitochondrial respiratory chain complex is decreased in liver tissue of patients and animal models with NAFLD[26,39].

A number of mechanisms can be considered to explain the mitochondrial dysfunction found in NAFLD patients and animal models. Possible mechanisms include (a) excessive reactive oxygen species (ROS) production, (b) increased TNF-α expression, and (c) altered PGC-1 expression.

Reactive oxygen species

MRC dysfunction can directly lead to the production of ROS. If electron flow is interrupted at any point in the respiratory chain, the preceding respiratory intermediates can transfer electrons to molecular oxygen to produce superoxide anions and hydrogen peroxide[40,41]. Malondialdehyde and 4-hydroxynonenal, two byproducts of lipid peroxidation, can inhibit mitochondrial cytochrome *c* oxidase by forming adducts with this enzyme. ROS-induced depletion in mtDNA can severely lower mitochondrial number and function leading to steatosis and liver lesions[34]. Such depletion can impair the synthesis of complexes Ⅰ, Ⅲ, Ⅳ and Ⅴ of the MRC, because mtDNA encodes for 13 of the MRC polypeptides. Evidence of oxidative stress has been found in patients with NASH[21]. Decreased activity of the MRC in ob/ob mice is in part attributable to the oxidative stress, because treatment of ob/ob mice with antioxidant MnTBAP, a mimic of manganese superoxide dismutase, improved the activity of several complexes of the MRC. Surprisingly, activity of complex Ⅱ, which polypeptides are only encoded by nuclear DNA, decreased even more in ob/ob mice treated with MnTBAP. Furthermore, liver histology improved markedly in mice treated with MnTBAP. MRC complexes' activity was reduced by 30% to 50% versus control activity. These complexes' activity was inversely correlated to blood TNF-α levels, body mass index, and HOMA index[26].

TNF-α

Another important factor to consider in the pathogenesis of mitochondrial dysfunction is TNF-α. High blood TNF-α levels have been found in patients with NASH[26,42,43]. In ob/ob mice TNF-α concentrations in liver tissue were some 20-fold higher than in normal mice[39]. The likely sources of the hepatic TNF-α are hepatocytes and Kupffer cells[44]. TNF-α induces mitochondrial swelling with a lighter matrix and a loss of septa. In addition, TNF-α induced swelling of the mitochondria causes a bursting of the mitochondrial membrane leading to an interference between MRC complexes Ⅰ and Ⅲ[45,46]. Anti-TNF-α treatment in ob/ob mice improve complex Ⅰ, Ⅱ, Ⅲ and Ⅴ activity, β-oxidation activity, and liver histology[39].

PGC-1

Nuclear receptors are pleiotropic regulators of glycolytic and oxidative metabolism[47]. Mitochondrial activity is transcriptionally controlled, in part, by the nuclear receptors and the peroxisome proliferator-activated

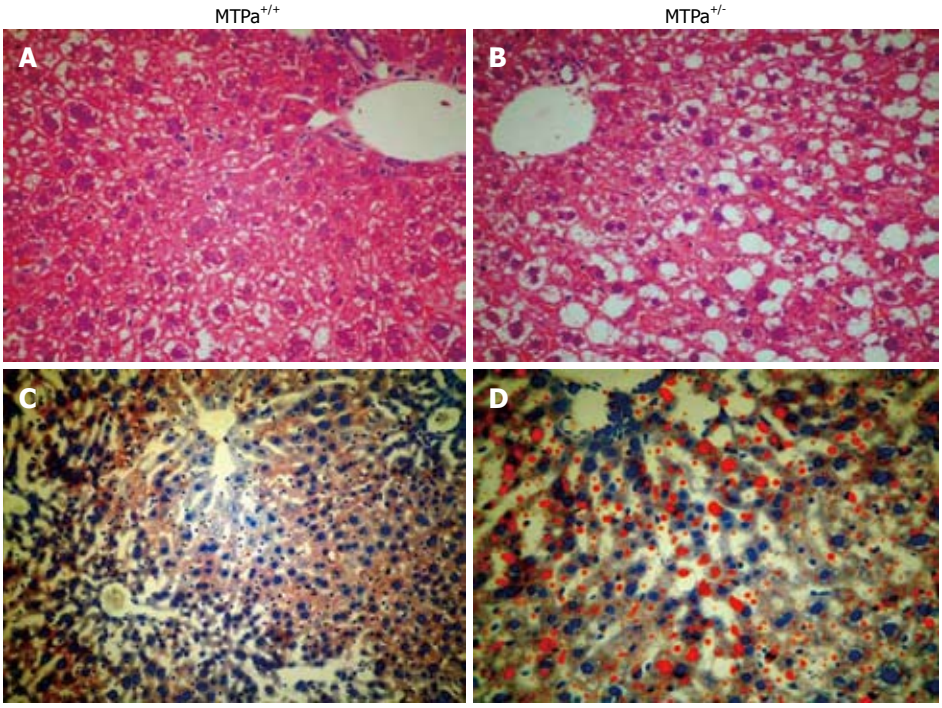


Figure 3 Representative liver sections obtained from control wild-type mice ($MTPa^{+/+}$) (**A** and **C**) and mice with a defect in mitochondrial trifunctional protein ($MTPa^{+/-}$) (**B** and **D**) littermates stained with hematoxylin-eosin (**A** and **B**) and oil red O (**C** and **D**) (20 ×). Reproduced with permission from *Gastroenterology*. 2005; 128: 1381-1390.

receptor-γ coactivator 1 (PGC-1)-related protein family such as PGC-1α and PGC-1β[48,49]. PGC-1α and PGC-1β are preferentially expressed in tissues with high oxidative capacity such as heart, skeletal muscle and brown adipose tissue, where they serve critical roles in the regulation of mitochondrial functional capacity and cellular energy metabolism[50-53] PGC-1α potently induces the expression of genes implicated in energy homeostasis in almost every cell type through known mitochondrial regulators such as the estrogen-related receptors (ERRs), peroxisome proliferator-activated receptor δ, or nuclear respiratory factor (NRF-1, 2)[48,49,54,55]. Overexpression of PGC-1α in skeletal muscle cells results in an increased energy expenditure, mitochondrial biogenesis[52,53], whereas loss of PGC-1α results in reduced muscle performance, cardiac defects, and other metabolic and behavioral defects[56,57]. Liver expresses low levels of PGC-1α and PGC-1β at normal condition, however, their expression is upregulated at fasting[58-60]. PGC-1α and PGC-1β activate hepatic fatty oxidation by inducing expression of PPARα[59,60]. Hepatocytes from PGC-1α deficient mice have diminished FAO activity and mitochondrial respiration rates[49].

CONCLUSION

Genetic, environmental and nutritional factors are involved in the pathogenesis of NAFLD, which is nowadays a major public health problem. In the absence of proven pharmacological therapy of NAFLD, it is critical to explore its pathogenesis and novel therapeutic pathways. In this Editorial, we have reviewed evidence that implicates mitochondrial dysfunction as a primary mechanism for development of NAFLD. Mitochondrial dysfunction may not only cause fat accumulation, but also may lead to the generation of ROS and cytokine production contributing to progression of NAFLD by inducing hepatic inflammation and fibrosis.

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TOPIC HIGHLIGHT

Jamal A Ibdah, MD, PhD, *Series Editor*

Role of the JNK signal transduction pathway in inflammatory bowel disease

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Abstract

The c-Jun NH2-terminal Kinase (JNK) pathway represents one sub-group of the mitogen-activated protein (MAP) kinases which plays an important role in various inflammatory diseases states, including inflammatory bowel disease (IBD). Significant progress towards understanding the function of the JNK signaling pathway has been achieved during the past few years. Blockade of the JNK pathway with JNK inhibitors in animal models of IBD lead to resolution of intestinal inflammation. Current data suggest specific JNK inhibitors hold promise as novel therapies in IBD.

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Key words: JNK pathway; Inflammatory bowel disease; Inflammation

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INTRODUCTION

Although our understanding of the pathogenesis of inflammatory bowel disorders (IBD), especially Crohn's disease (CD) and ulcerative colitis (UC) has greatly improved, the specific causes are still not known. Cytokines such as TNF-α, IL-1 and IFN-γ play an important role in the pathogenesis of IBD[1,2]. Elucidating the mechanisms of cytokine induced inflammation in IBD could lead to novel therapies. Recent studies have focused on the identification of intracellular signaling pathways and transcription factors through which cytokines mediate their effects. Mitogen-activated protein kinases (MAPKs) are components of the signaling cascades where diverse extracellular stimuli converge to initiate inflammatory cellular responses. MAPKs are made of three subgroups-p42/44 extracellular signaling kinase (ERK), Jun-N-terminal Kinase (JNK), and p38 MAP Kinase[3]. Recent studies have highlighted the importance of the JNK pathway in IBD. This review will focus on the role of the JNK signaling pathway in IBD.

THE JNK-MAPK PATHWAY

The mammalian JNK were initially called stress activated protein kinase (SAPK) because they were activated by a variety of environmental stresses[3,4]. Later, the JNK pathway was also discovered to respond to cytokines, such as TNF-α and IL-1, and growth factors. JNK is a multi-factorial kinase involved in several physiological and pathological processes. Specific stimuli trigger the activation of MAP3Ks, which then phosphorylate and activate the MAP2K isoforms MKK4 and MKK7, which in turn phosphorylate and activate JNK[5]. JNK was discovered to phosphorylate c-Jun at the NH2-terminal Ser63 and 73 residues, and thus termed JNK. However, several recent studies have shown that JNK can phosphorylate a variety of substrates, including additional transcription factors and some non-nuclear proteins[3,6]. In addition to c-Jun, JNK can phosphorylate transcription factors such as JunB, JunD, c-fos, ATF2 and ATF3. These transcription factors along with c-Jun, make up the Activator Protein-1 transcription factor (AP-1), which regulates the expression of several stress-responsive genes. The JNK class of enzymes comprises of three main types: JNK1, JNK2, and JNK3[4,7]. The first two are ubiquitous, whereas the third is restricted to the brain, heart and testis. Differential splicing and exon usage results in multiple isoforms of JNK1, 2 and 3 genes. Each JNK is expressed as a short form (46 kDa) and long form (54 kDa)[3]. The alternative forms of each JNK1, 2, and 3 appear to differ in their ability of bind and phosphorylate different substrate proteins. Targeted gene disruption of each JNK has also defined differential functions for JNK1, JNK2 and JNK3 in many different cell types[7]. Deletion of JNK1 or JNK2 resulted in defective T cell differentiation and activation[8].

ROLE OF JNK IN IBD

The JNK pathway is considered to be a potentially relevant target for therapy inflammatory disease states. JNK regulates the maturation and activity of T cells and synthesis of pro-inflammatory cytokines such as interleukin-2 (IL-2), IL-6 and TNF-α. Several recent studies have demonstrated the importance of JNK pathway in chronic inflammatory disorders involving the expression of specific proteases and cytokines. For example, JNK pathway appears to be involved in the expression of TNF-α in rheumatoid arthritis[9]. JNK inhibitors such as SP600125 protected mice from joint damage in rheumatoid arthritis animal models[9]. Additionally, the JNK pathway also plays a role in artherosclerosis[10,11]. These findings led to the investigation of the role of JNK pathway in intestinal inflammation.

JNK activation in human intestine in patients with IBD was shown in 4 studies[12,13]. Increased activation of JNK along with ERK and p38 MAPK in human colonic tissue from 27 patients with moderate to severe CD or UC was first shown by Waetzig et al. Hommes *et al* also noted increased activation of p38 and JNK in their study involving 12 patients. Mistsuyama *et al* subsequently confirmed these findings in their recent study. They examined whether JNK phosphorylation was greater in sites of active inflammation compared to normal intestine in patients with IBD. Both ELISA and immunostaining demonstrated that JNK was highly activated in colonic tissue with active disease. Phospho-JNK was present in the intestinal cells, macrophages and lymphocytes, localized pre-dominantly in the nucleus. These findings validated the results from *in-vitro* cell culture studies[14]. Interestingly, increased JNK activation has also been shown in steroid-resistant patients[15]. The significance of this finding is currently unclear. The detection of enhanced activation of JNK in intestinal tissue in patients with IBD may serve as a diagnostic tool for early recognition of steroid unresponsiveness. Role of the different isoforms of JNK in IBD was investigated in a recent study[16]. Deletion of either JNK1 or JNK2 did not prevent the development of colitis in animals. However, deletion of JNK2 was associated with deterioration of disease activity. Further studies examining the role of different isoforms of JNK in IBD are needed.

The role of JNK inhibitors as potential therapies for IBD has been studied in both animal models of IBD and in humans. There are at least 40 different small-molecule JNK inhibitors that have either published or patented[3]. These inhibitors either affect JNK signaling pathway indirectly (e.g. CEP 1347) or block the catalytic domain of JNK (e.g. SP 600125). Unfortunately, most of these compounds only have a moderate specificity for JNK and may also interfere with other signaling pathways. Peptide inhibitors of JNK pathway, which have a higher specificity for their targets, are currently being developed. However, one of the major obstacles with peptide drugs is their rapid degradation and difficulty with delivery across cell membranes. These obstacles have been reportedly overcome by a recently described cell-permeable peptide that contains the JNK-binding domain of human c-Jun. Two studies assessed the effect of JNK inhibitor, SP 600125, on dextran sodium sulphate (DSS) colitis animal model[12,17]. SP 600125 is a reversible ATP-competitive inhibitor of protein kinases. It targets all the three different isoforms of JNK. At higher concentrations, it inhibits other protein kinases upstream of JNK (namely MKK3, and MKK6). One study evaluated SP 600125 in a rat model (Sprague-Dawley rats) of DSS colitis while the other used a mice model (C57BL/6) of DSS colitis. Both studies demonstrated the activation of JNK pathway in inflamed intestinal tissue in DSS induced colitis. JNK inhibition showed a marked protective effect against experimental colonic injury in animals. Specifically, treatment with SP600125 led to attenuation of weight loss and macroscopic damage. A beneficial effect was also noted on the histological severity of colitis. Destruction of the epithelial layer and glandular architecture, inflammatory infiltrates in the lamina propria, and edema of the submucosa in the colon was less severe in the SP600125 treated animals. Treatment with SP 600125 also resulted in a significant reduction in the levels of TNF-α, IL-6 and IFN-γ. Additionally, SP 600125 inhibited cytokine production by activated CD3/CD28 mesenteric lymphocytes[17]. One major limitation of these studies is that a more specific inhibitor of JNK was not investigated. Animal studies utilizing a peptide inhibitor or SiRNA against the JNK pathway are needed. Human studies have also suggested similar benefits of JNK blockade to those seen in animals. CNI-1493, a guanylhydrazone that inhibits the phosphorylation of both JNK and p38 MAP kinase, was studied in an open- label pilot study in 12 patients with moderate to severe Crohn's disease. Two different doses of CNI-1493 (8 or 25 mg/m^2) were given intravenously once daily for 12 d. A significant change in CDAI from baseline was noted at wk 2 and persisted up to wk 16. CRP levels decreased significantly during the first weeks of treatment. Endoscopic improvement was observed in all but one patient. Five patients had active fistulizing CD, and closure of the fistula was observed in 4 patients. A steroid sparing effect was seen in 89% of patients maintained on steroids. Additionally, CD-related arthralgia/arthritis resolved in all patients. Although the small sample size in this study precludes any significant conclusions, this study suggests CNI-1493 has significant therapeutic potential in CD. Further studies using JNK specific inhibitors in IBD are currently needed.

CONCLUSION

The JNK pathway plays an important role in various inflammatory disorders. Recent data suggest that JNK activation plays an important role in the intestinal inflammation in patients with IBD. However, the role of the different JNK isoforms in IBD has not been elucidated. Additionally, the mechanism by which JNK activation leads to intestinal inflammation is unclear and deserves further study. Cross talk of JNK pathway with other signaling pathways also needs to be investigated. Recent studies suggest a role for JNK blockade in IBD therapy. However, JNK inhibitors which could also inhibit other kinases were used. Studies using JNK specific inhibitors (e.g. peptide inhibitors) are needed. To increase

the likelihood of success, it may be important to develop isoform-specific JNK inhibitors, as they are likely to have increased efficacy and specificity resulting in fewer potential side effects.

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Methylation of TIMP3 in esophageal squamous cell carcinoma

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Abstract

AIM: To measure the frequency of DNA methylation of the tissue inhibitor of metalloproteinase 3 (TIMP3) promoter and relate this to any change of gene expression in esophageal squamous cell carcinoma in patients from a region of high incidence in China.

METHODS: Cancer cell lines were treated with or without the demethylating reagent 5-aza-2′-deoxycytidine. Methylation of the TIMP3 promoter was assessed in three regions by melt curve analysis and its expression was assessed by real-time RT-PCR. Tumors and proximal resection margins were obtained from 64 patients with esophageal squamous cell carcinoma from a region of high incidence in China. Methylation was assessed by melt curve analysis and expression by immunohistochemistry.

RESULTS: Methylation in one of the three promoter regions assessed correlated with gene silencing in esophageal cell lines. A degree of methylation of TIMP3 was found in only four esophageal squamous cell carcinomas, and partial loss of TIMP3 protein expression in just one.

CONCLUSION: Methylation and loss of expression of TIMP3 occurs infrequently in esophageal squamous cell carcinoma in a region of high incidence in China.

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Key words: Esophageal squamous cell carcinoma; Immunohistochemistry; Methylation; TIMP3

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INTRODUCTION

Tumor invasion, metastasis and angiogenesis require proteolysis and remodeling of basement membranes and extracellular matrix (ECM) by enzymes such as matrix metalloproteinases (MMPs). The MMPs can degrade all components of the ECM, which makes regulation of these enzymes important in the development and dissemination of cancer. Many factors, including inflammatory cytokines, growth factors, hormones, and cell-cell and cell-matrix interactions can alter the transcription of the MMP genes[1], while enzymes can be specifically inhibited by TIMPs, which bind covalently to the active site of the enzyme. Altered expression of MMPs is associated with a poor prognosis in a range of solid tumors[2].

TIMP3, which binds to the ECM, is a multifunctional secreted protein with properties not just limited to the inhibition of MMPs. These roles of TIMP3 in cancer progression are highlighted in reports that its vector-mediated expression in cancer cells reduces metastasis[3], induces apoptosis, augments drug sensitivity in prostate cancer cell lines[4], and inhibits tumor growth in lung cancer cells in an *in vivo* model[5].

A reduction in TIMP3 expression has been reported to correlate with poor outcome in a number of cancers. DNA methylation of gene promoters is one method of silencing transcription, and TIMP3 methylation has been noted in a wide range of tumors. Reports indicate that the reduced expression of TIMP3 is a common occurrence in esophageal adenocarcinoma (EAC), is associated with methylation of the promoter, and correlates with poor outcome[6,7]. TIMP3 is also frequently methylated in Barrett's esophagus (BE), and has been investigated as a prognostic indicator for progression to EAC[8,9]. In contrast to the many studies of EAC, there is a study of esophageal squamous cell carcinoma (ESCC) in a cohort of patients

from Japan that has shown a decrease in TIMP3 protein expression, as measured by immunohistochemistry (IHC), which correlates with invasive activity and metastasis[10]. However, the mechanism responsible for the reduction of this expression has not been investigated. This study aimed to measure the frequency of methylation of TIMP3 in ESCC in patients from a region of high incidence in China, and to determine if this correlated with a reduction of TIMP3 expression.

MATERIALS AND METHODS

Patient samples

Primary tumors and, when available, non-cancerous proximal resection margins from 64 consecutive patients undergoing resection for ESSC at the Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University, China were preserved in RNAlater (Ambion, Austin, TX, USA). Patient gender, age at the time of operation, and tumor stage, differentiation and volume were recorded (Table 1). Survival data were available for 45 patients. The study complied with all appropriate institutional guidelines.

Table 1 Characteristics of patients with ESCC

Variable	*n*
Male	45
Female	19
Age, median (range)	57 (42-76)
Males	57 (42-70)
Females	62 (49-76)
Tumor volume (cm^3), median (range)	57 (4-300)
Histological differentiation	
Well/moderate	57
Poor	4
Not recorded	3
Tumor stage	
T1N0M0	3
T2N0M0	9
T2N1M0	4
T3N0M0	29
T3N1M0	10
T3N1M1	1
T4N0M0	2
T4N0M1	1
T4N1M0	2
Not recorded	3

Demethylation of cell lines with 5-aza-2'-deoxycytidine (aza-dC)

Demethylation studies of TIMP3 were performed on nine cancer cell lines, OE19, OE21, OE33, TE7, DU145, LNCAP, T47D, ZR75.1, and KCL22. The cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37℃ in 5% CO_2. Cells were seeded into flasks and cultured for 24 h before they were treated with 0 or 1 μmol/L aza-dC (Sigma-Aldrich, Sydney, NSW, Australia). To determine the length of time required for the cells to undergo at least two divisions in the presence of aza-dC, selected cell lines were labeled with PKH-26 (Sigma-Aldrich), as described previously[11]. Cell lines were treated for either 72 or 96 (OE19) h with 0 or 1 μmol/L aza-dC. The medium was then replaced with fresh medium not containing aza-dC, and the cells incubated for a further 24 h before harvesting.

Preparation of bisulfite-modified DNA

Genomic DNA was isolated from normal donor lymphocytes, cultured cells, and RNAlater-stabilized tissues as previously described[11]. DNA (2 μg) was bisulfite-modified as previously described[11], except that bisulfite-modified DNA was purified using an UltraClean PCR Clean-up DNA Purification Kit (MO BIO Laboratories, Carlsbad, CA, USA), and resuspended in 100 μL ultra pure water (Fisher Biotec Australia, Wembley, WA, Australia).

Methylation analysis of the TIMP3 promoter

Bisulfite-modified DNA was amplified using primers that amplified three overlapping regions designated R1, R2 and R3 (Figure 1). The primer sets did not discriminate between methylated and unmethylated sequences. The primers and PCR conditions were specific for bisulfite-modified DNA, and did not amplify unmodified DNA. All methylation-analysis PCRs were performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) in a final volume of 15 μL, containing 1 μL bisulfite-modified DNA and a final concentration of 0.5 μmol/L forward and reverse primers (GeneWorks, Therbarton, SA, Australia) (Table 2). Bisulfite-modified lymphocyte and CpG methylase-treated lymphocyte DNA were included in each PCR run, and served as unmethylated and methylated controls, respectively. Reactions were incubated in a Rotor-Gene 3000 (RG-3000) (Corbett Life Science, Sydney, NSW, Australia) at 95℃ for 15 min, then 45 cycles of 95℃ for 30 s and 55℃ for 60 s, and a final extension of 72℃ for 4 min. Methylation was determined by analyzing the melt curve of the PCR product at the end of the amplification cycle. The temperature was ramped from 60 to 95℃, rising 0.5 or 1℃ at each step, waiting 30 s on the first step, then 5 s for each step thereafter. The dF/dT was determined for each PCR product using the RG-3000 application software v6 (Corbett Life Science). The dF/dT curves of the samples were compared to those of the unmethylated and methylated controls. A sample was considered methylated when there was a shift in its dF/dT curve away from that of the unmethylated control. The degree of methylation was graded as -, +, ++, +++ according to the degree of the shift to the right, as assessed by two independent observers.

Expression of TIMP3 mRNA in cell lines by quantitative real-time RT-PCR

Cell line RNA was isolated using an RNeasy kit with on-column DNase I digestion (Qiagen). The cDNA was synthesized from 2 μg RNA using SuperScript Ⅱ (Invitrogen, Mount Waverly, Vic, Australia). Quantitative real-time RT-PCR (qRT-PCR) was performed using the QuantiTect SYBR Green PCR Kit in a final volume of 10 μL, containing 1 μL cDNA and a final concentration of 0.5 μmol/L forward and reverse primers (Table 2). Triplicate reactions were incubated in an RG-3000 at 95℃ for 15 min, then 45 cycles of 95℃ for 30 s and 60℃ for

Table 2 Primer sequences for methylation analysis and quantitative real-time RT-PCR

	Forward primer	Reverse primer	Length (bp)
TIMP3 R1	TTTGGGTTAGAGATATTTAGTGGTT	RAATCRTTCAAATCCTTATAAAAAATAATA	175
TIMP3 R2	GGYGGTATTATTTTTTATAAGGATTTG	AAACCCCRCCTCRAACTATTAAA	158
TIMP3 R3	TTTGAGGGGGYGGGTTTTAATAGTT	AACRACCTCCCRACGAAAAAACAAA	174
TIMP3 qRT-PCR	CCAGGACGCCTTCTGCAAC	CCTCCTTTACCAGCTTCTTCCC	71

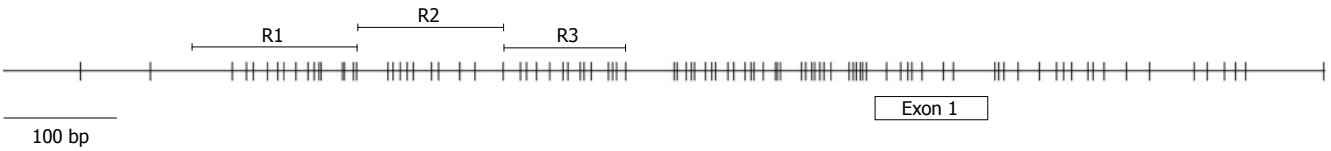


Figure 1 Schematic representation of the TIMP3 promoter. The schematic was generated by downloading the TIMP3 CpG island genetic element from http://genome.ucsc.edu/, using the Human March 2006 Freeze. The relative location of TIMP3 exon 1 is indicated. Each vertical line represents the location of a CpG. The three overlapping regions (R1, R2 and R3) amplified for methylation analysis are shown.

60 s, and a final extension of 72℃ for 4 min. The PCR products were electrophoresed on 1.5% (w/v) agarose gels and stained with ethidium bromide to confirm expected product sizes. The expression of TIMP3 was normalized to that of porphobilinogen deaminase (PBGD)[11].

Analysis of TIMP3 expression in ESCC by immunohistochemistry

Primary tumors and non-cancerous proximal resection margins preserved in RNAlater were fixed in formalin and embedded in paraffin using routine histopathology protocols. Sections (4 μm) of the formalin-fixed paraffin-embedded tissue were mounted on to polylysine-coated slides, de-waxed and rehydrated. Antigen retrieval was performed by heating the sections for 5 min in 10 mmol/L citrate buffer (pH 6) in a microwave pressure cooker. After cooling to below 30℃, sections were immunostained at room temperature using a Dako Autostainer Plus (Dako, Glostrup, Denmark). Sections were incubated for 60 min with a 1:750 dilution of mouse anti-human TIMP3 monoclonal antibody (Chemicon International, Temecula, CA, USA), and then with the MACH 4 Universal HRP Polymer (Biocare Medical, Concord, CA, USA). Visualization was performed with liquid 3, 3-diaminobenzidine (Dako) as the chromogen. Sections were counterstained with Meyer's hematoxylin.

Statistical analysis

Statistical comparison of the expression of TIMP3 mRNA following treatment with and without aza-dC for each cell line was performed using unpaired *t* tests. All statistical analysis was performed using InStat version 3.0a (GraphPad Software, San Diego, CA, USA).

RESULTS

Expression and methylation of TIMP3 in cell lines

The expression of TIMP3 mRNA was measured by qRT-PCR in cell lines without (0 μmol/L) and with (1 μmol/L) aza-dC demethylation treatment (Figure 2). Without aza-dC treatment, no TIMP3 expression was detected in

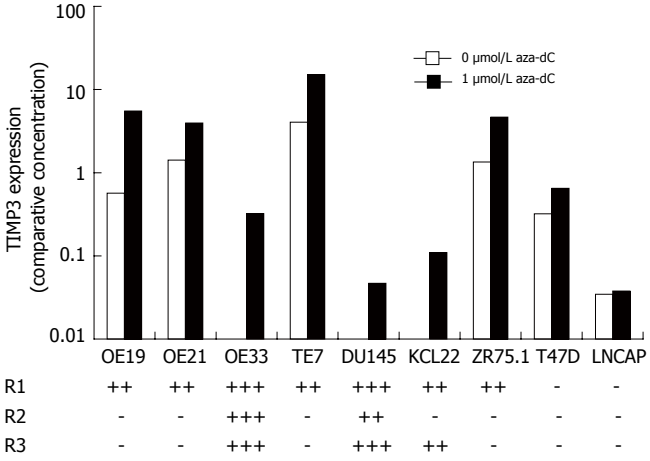


Figure 2 Expression and methylation of TIMP3 in cancer cell lines. The level of TIMP3 mRNA in cell lines treated with either 0 or 1 μmol/L aza-dC was determined by qRT-PCR and normalized using PBGD. Methylation was determined by melt curve analysis in three overlapping regions R1, R2 and R3, and is summarized.

OE33, DU145 and KCL22 cells, but was detected in all other cell lines tested. A significant increase in expression was induced by aza-dC in all cell lines except LNCAP.

The methylation in three overlapping regions of the TIMP3 CpG island (Figure 1) was evaluated by melt curve analysis and is summarized in Figure 2. Methylation in R1 was observed in OE19, OE21, OE33, TE7, DU145, KCL22, and ZR75.1 cells. Methylation in R2 was observed in OE33 and DU145 cells. Methylation in R3 was observed in OE33, DU145 and KCL22, the cell lines that did not express TIMP3 prior to treatment with aza-dC. Treatment with aza-dC caused a reduction in methylation in all regions in which methylation was observed (Figure 3). These data suggested that in the cell lines evaluated in this study, methylation in R3 was the one associated with transcriptional silencing of TIMP3 expression.

Expression and methylation of TIMP3 in ESCC

Methylation of the TIMP3 CpG island was evaluated in

Table 3 TIMP3 methylation and expression in methylated ESCC samples

Patient	M/F	Age	Tumor			Methylation			Expression
			Volume (cm^3)	Differentiation	Stage	R1	R2	R3	
28	F	65	60	Moderate	T3N1M1	++	-	-	Strong heterogeneous
41	M	65	30	Moderate	T3N0M0	++	-	++	Strong heterogeneous
44	M	66	18	Moderate	T3N0M0	++	-	++	Strong heterogeneous
75	F	72	27	Moderate	T3N1M0	++	+	++	Focal loss

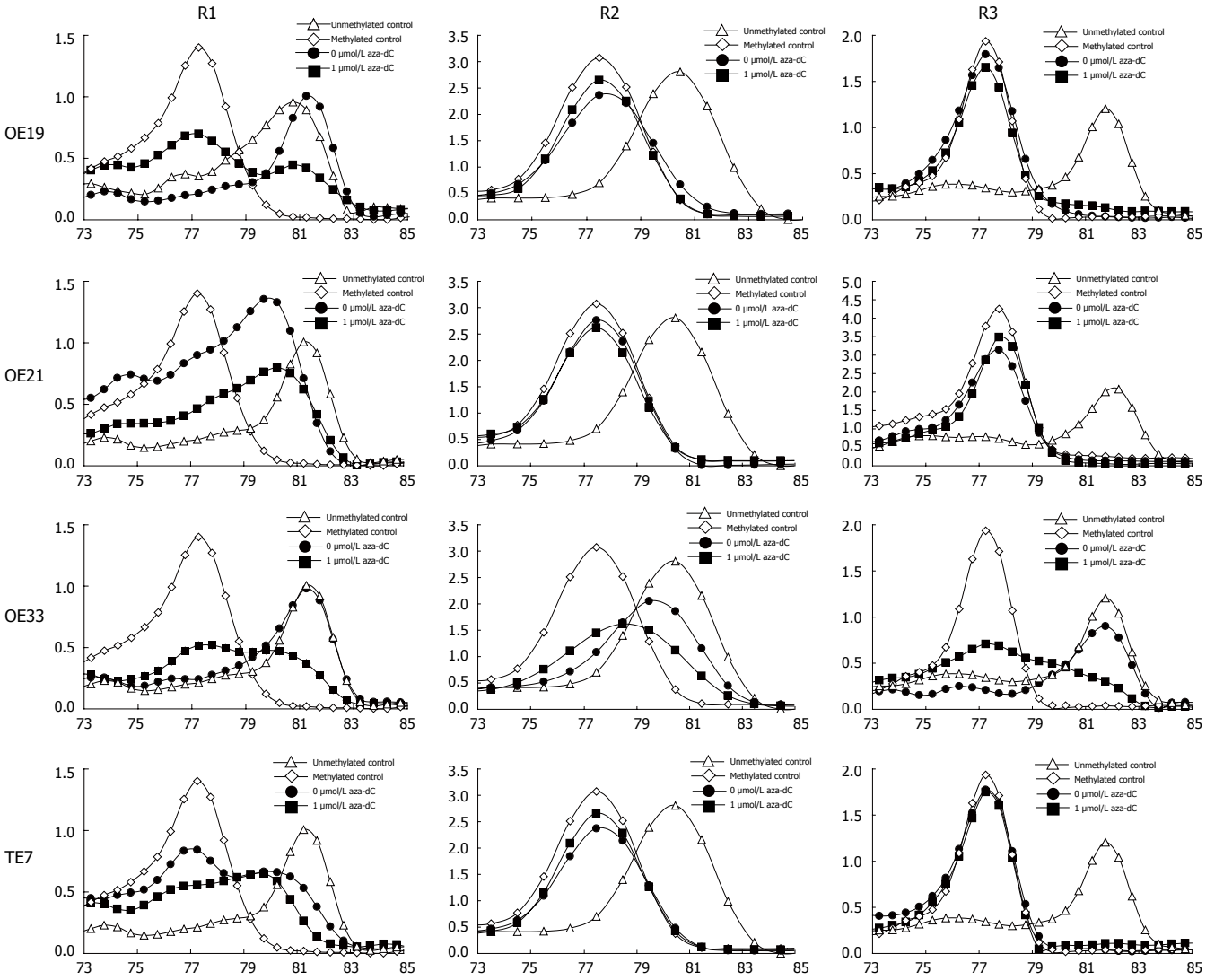


Figure 3 Methylation of TIMP3 in esophageal cancer cell lines. The X axis is temperature, the Y axis is fluorescence dF/dT.

tissues resected from patients with ESCC. Methylation was not detected in R1, R2 or R3 in the cancer-free proximal resection margins from these patients. However, methylation was detected in tumors from 4/64 (6%) patients. In the four tumors in which methylation was detected, low levels of methylation were present in R1 but not R2 or R3 in one patient, in R1 and R3 but not R2 in two patients, and in all three regions in one patient (Figure 4). TIMP3 protein expression was assessed in patient tissues by IHC. Strong heterogeneous cytoplasmic expression of TIMP3 was limited to the basal layers of the squamous mucosa (Figure 5A). All ESCCs that were unmethylated in all three regions analyzed displayed strong heterogeneous cytoplasmic staining of TIMP3 in cancer cells (Figure 5B and C). A significant loss of TIMP3 staining was not observed in the ESCCs that were methylated in either R1 or R1 and R3. By contrast, focal loss of TIMP3 staining was observed in the tumor that was methylated in all three regions (Figure 5D). TIMP3 methylation or loss of expression was not associated with patient age, gender, tumor volume, differentiation or stage (Table 3), or survival.

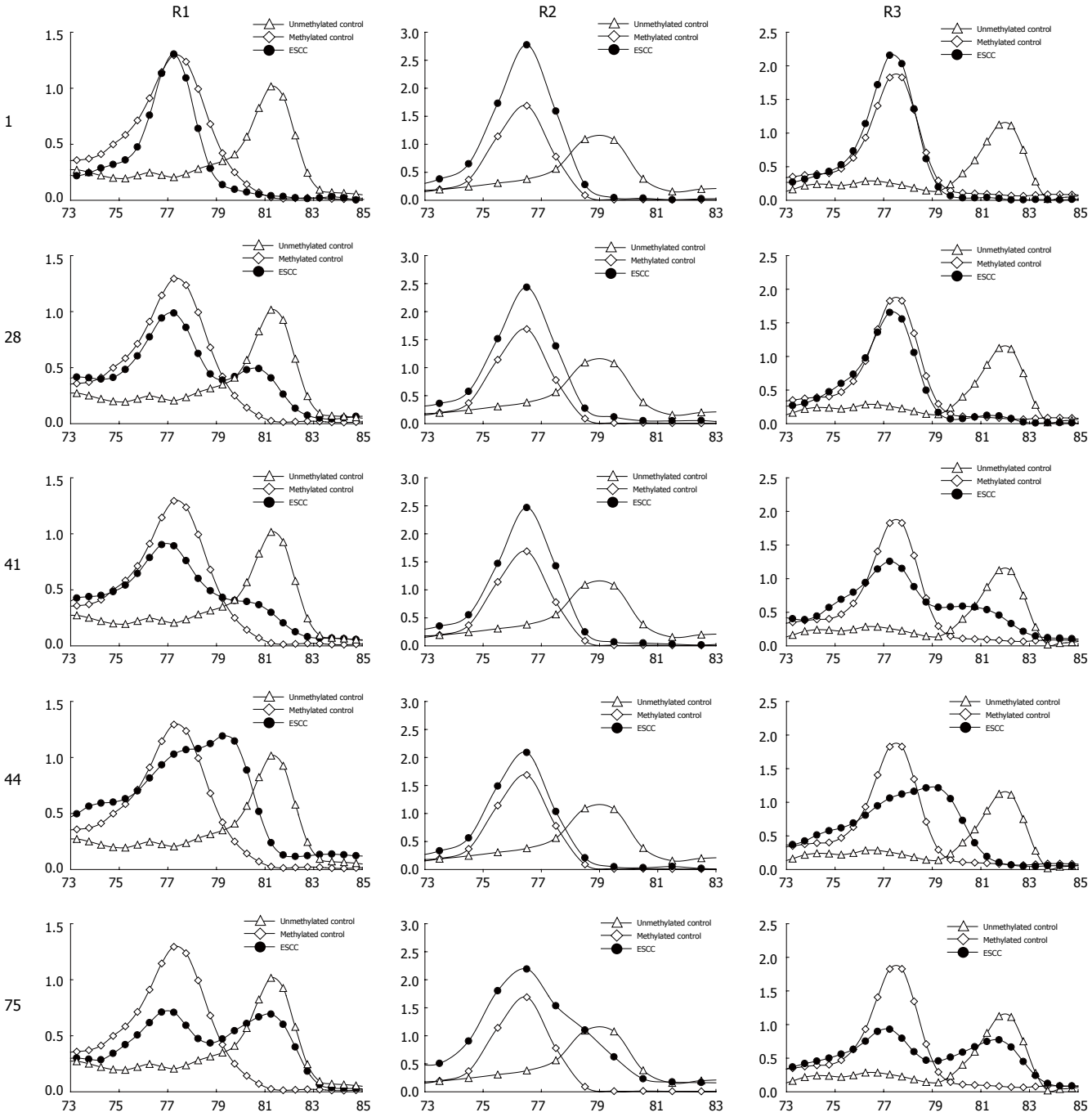


Figure 4 Methylation of TIMP3 in ESCC samples. The X axis is temperature, the Y axis is fluorescence dF/dT.

DISCUSSION

TIMP3, as an inhibitor of the proteolytic activity of MMPs, is believed to reduce tumor invasiveness and metastasis. Down-regulation of TIMP3 expression occurs in a number of cancers and has been linked to a poor outcome. One possible mechanism for the reduction in TIMP3 expression is DNA methylation of its promoter region. We first determined which region of the promoter CpG was associated with gene silencing by measuring gene expression and methylation in three overlapping regions of the TIMP3 CpG island in nine cancer cell lines. We found that methylation in only one of these regions appeared to be important in gene silencing. We then measured methylation and expression of TIMP3 in ESCC specimens resected from 64 patients from an area of China that has a very high incidence of this disease. Methylation was present in any of these three regions in just four of the 64 ESCC samples examined. Significant focal loss of TIMP3 protein expression was only observed in the tumor of one patient with ESCC, which was methylated in all three regions.

TIMP3 not only inhibits MMPs but also inhibits tumor growth and angiogenesis[12,13], and induces apoptosis in cancer cells[4,14]. Clinical studies have reported a reduction in TIMP3 expression that correlates with clinical

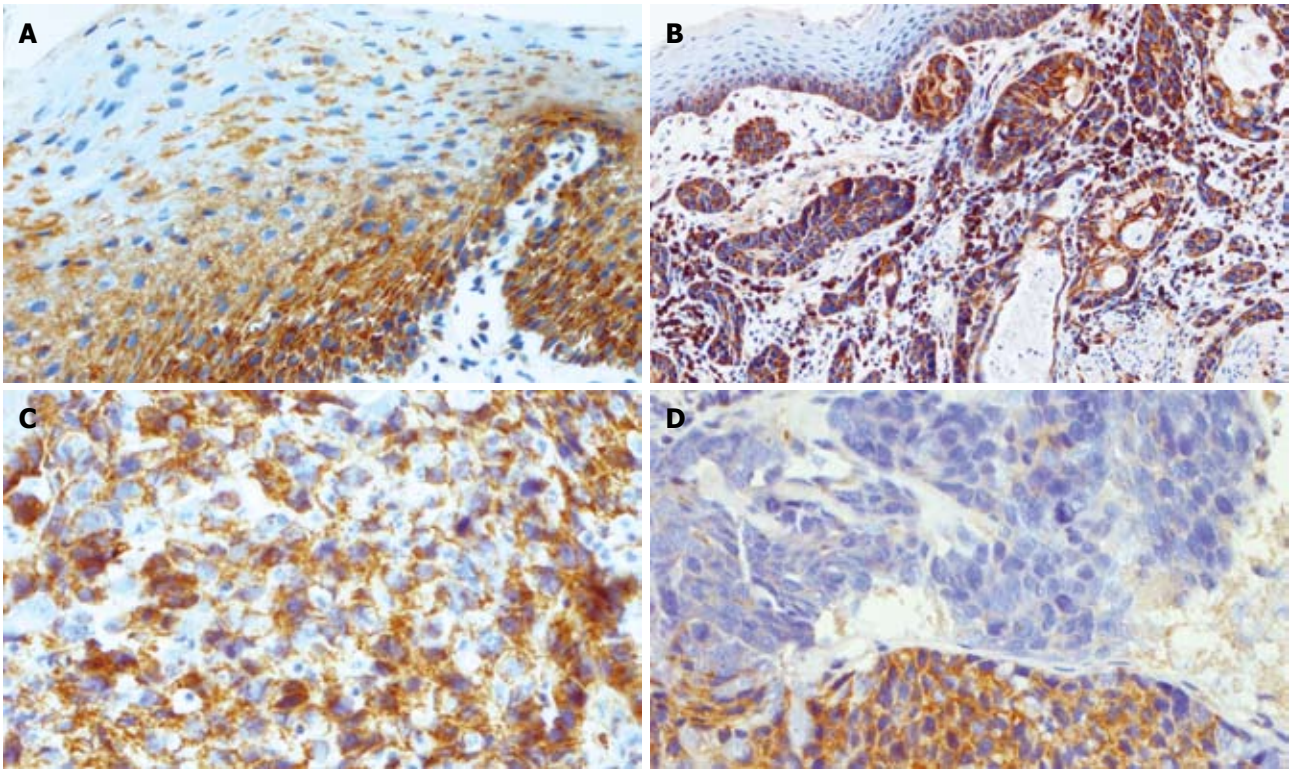


Figure 5 Expression of TIMP3 in esophageal tissues. **A**: Strong heterogeneous cytoplasmic expression of TIMP3 was limited to the basal layers in normal squamous mucosa; **B**: TIMP3 expression in unmethylated ESCC underlying normal squamous mucosa; **C**: Unmethylated ESCC showing strong heterogeneous staining; **D**: Methylated ESCC showing the region of reduced expression.

parameters in a number of cancers, including colon[15], lung[16,17] and breast[18,19] cancer and choriocarcinoma[20]. Methylation of the TIMP3 promoter is associated with silencing of transcription and has been noted in tumors of the thyroid[21,22], lung[16,17], bladder[23,24], stomach[25,26], uterus[27,28], bone[29], breast[30], liver[31] and colon[32,33].

Several studies have suggested that there is regional variation in TIMP3 expression within a tumor. Powe *et al* reported that TIMP3 mRNA expression decreases at the invasive edge of poorly differentiated colon carcinoma, and suggested that a regional loss of TIMP3 may contribute to increased invasiveness[34]. In normal esophagus, TIMP3 expression is restricted to the cytoplasm of basal, parabasal and stromal cells. Darnton *et al*[6] reported that TIMP3 protein expression was observed in all of 79 EACs examined, but staining intensity varied throughout the tumor. Whilst superficial areas of the tumors were always intensely stained, reduced expression was observed in central regions and variable expression, from pale to intense, was observed at the invading edges. Reduced TIMP3 expression was more often observed in an advanced tumor stage and was associated with reduced patient survival, but not lymph node positivity or tumor differentiation. TIMP3 mRNA and methylation were detected in 16/16 and 19/21 primary EACs, respectively, but correlation with reduced staining was not reported[6].

Most interest in methylation in cancer has focused on regions with relatively high densities of CpG islands, which are associated with gene promoter regions. It is common for methylation to vary between different regions of a particular CpG island when comparing DNA isolated from different cell lines or from different human tissues. For some genes, methylation of particular regions of the CpG island appears to be more important as a cause of silencing than methylation of other regions. Using established cancer cell lines, we analyzed three overlapping regions of the CpG island and correlated the methylation in each with expression, to determine if some regions of the island were more important than others in regulating gene expression.

The first detailed analysis of DNA methylation of the CpG island at the TIMP3 promoter was performed by Bachman *et al*[35]. They have demonstrated methylation in R1 in colon cancer cell lines, irrespective of whether or not these cells expressed TIMP3 mRNA. Their data are consistent with our findings in cell lines, and together, these findings suggest that methylation in this region by itself is not sufficient for transcriptional silencing. Bachman *et al* have observed dense methylation in R2 and R3 in cell lines that do not express TIMP3, and less methylation in these two regions in cell lines that do express the TIMP3 transcript. Significantly, our results demonstrated that in cell lines, complete silencing of TIMP3 transcription was only observed when there was significant methylation in R3, and that this complete silencing occurred even though there was no detectable methylation in R2.

A consistent characteristic of methylation of the TIMP3 CpG island that we observed was that methylation in R2 only occurred when there was methylation in R3, which in turn only occurred when there was methylation in R1. These findings indicate that aberrant methylation in the TIMP3 CpG island is progressive, beginning in

R1, before progressing to R3 and then R2. Progressive methylation has been observed in the p16 CpG island of primary human mammary epithelial cells escaping from growth arrest[36]. Regional methylation within a CpG island that is sufficient to silence transcription has also been reported for other genes, including MGMT[37] and MT3[11].

More than 90% of esophageal cancers are either adenocarcinomas or squamous cell carcinomas. In EAC, a number of reports have indicated that reduced expression of TIMP3 is common, and is associated with methylation and poor outcome. Darnton *et al* reported methylation of TIMP3 in 90% of EAC and 72% of BE patients, and that reduced expression of TIMP3 protein in EAC is associated with increased tumor invasiveness and reduced patient survival[6]. By contrast, Brock *et al*[38] reported that only 19% of EAC tissues showed TIMP3 methylation, and that this by itself was not a prognostic indicator. Eads *et al* reported TIMP3 methylation in 86% of EAC, and in 78% of dysplastic BE patients, and low methylation in 33% of metaplastic BE patients[7]. Clement *et al* found that TIMP3 was methylated in 65% of EAC patients, and that a significantly higher percentage of patients with BE who progressed to EAC had methylated TIMP3 than those who did not progress, which suggests its value as a prognostic indicator in BE[8]. Schulman *et al*, however, reported TIMP3 methylation in 56% of EAC and 59% of BE tissues, and concluded that hypermethylation of TIMP3 is not an independent risk factor for progression from BE to EAC[9].

In contrast to the many studies of TIMP3 in EAC, there is only one in ESCC. Miyazaki *et al*[10] reported that in 90 patients who underwent surgery for ESCC at Gunma University Hospital (Japan), TIMP3 protein expression was always observed in cells in the shallow areas of the tumor, but was not expressed by cells at the invasive front of the same tumor. The percentage of cells with reduced TIMP3 expression at the invasive front varied between patients; in 33% of patients, 80% of the cells expressed TIMP3; in 30% of patients, 30%-80% of the cells expressed TIMP3; and in 37% of patients, < 30% of the cells expressed TIMP3. The patients that had the greatest percentage of cells with reduced expression had significantly reduced postoperative survival, increased depth of invasion, more metastatic lymph nodes, and higher disease stage. However, they did not analyze methylation of TIMP3. The low frequency of methylation that we observed in ESCC was not due to our methods, as we have found methylation in 88% (22/25) of EAC patients (unpublished observations); this is consistent with other reports of this cancer[7-9]. There is no obvious explanation for the discrepancy between our results in this Chinese cohort and the Japanese study. The disease may be biologically different, perhaps due to lifestyle, environmental or genetic differences. Further studies are required to investigate this issue.

We found that methylation of the TIMP3 CpG island was detectable in only 4% (4/64) of ESCC patients from a region in China that has a high incidence of this disease. The number of patients with methylation in R3, which we showed in cell lines correlated with transcriptional silencing, was 3/64. We found a loss of TIMP3 expression by immunohistochemistry in only one patient, and then this was only a focal loss. There is no obvious explanation for the presence of TIMP3 protein in patients with R3 methylation. Our results clearly showed that the loss of expression or methylation of TIMP3 is uncommon in ESCC from this region of China.

ACKNOWLEDGMENTS

We thank Dr. Yutaka Shimada for the generous gift of several of the cell lines used in this study.

COMMENTS

Background

Increased knowledge of the underlying biology of the cancer may lead to improvements in diagnosis and treatment. TIMP3 inhibits matrix metalloproteinases (MMPs), which can inhibit tumor growth and metastasis. There is one report suggesting that TIMP3 is reduced in esophageal squamous cell carcinoma (ESCC). We measured the frequency of methylation of TIMP3 in ESCC in patients from a region of high incidence in China, and determined if this correlated with a reduction of TIMP3 expression.

Research frontiers

Methylation which silences genes is generally found in regions with relatively high densities of CpG, known as CpG islands, which are associated with gene promoter regions. Measuring methylation in these critical regions of DNA may assist in diagnosis or prognosis in cancer.

Innovations and breakthroughs

We defined the region of the TIMP3 CpG island which is associated with the silencing of the gene. We found that reduction in expression of TIMP3 is uncommon in ESCC from a region of high incidence in China, and correspondingly methylation was infrequent as well.

Terminology

MMPs: enzymes which break down and reshape the molecules which surround a cell and hold it in its place. Methylation of DNA: one way to silence a gene. Gene promoter: the part of the DNA where the cell begins to make gene product.

Peer review

This is an excellent and well written contribution.

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Clinical significance of type VI pit pattern subclassification in determining the depth of invasion of colorectal neoplasms

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Abstract

AIM: To clarify whether subclassification of the type VI pit pattern on the basis of magnifying colonoscopy findings is useful in determining the type and depth of invasion of colorectal neoplasms.

METHODS: We retrospectively analyzed 272 colorectal neoplasms (117 dysplasias and 155 submucosal invasive carcinomas; 228 patients) with a type V pit pattern [type VI, *n* = 202; type VN, *n* = 70 (Kudo and Tsuruta classification system)]. We divided lesions with a type VI pit pattern into two subclasses, mildly irregular lesions and severely irregular lesions, according to the prominent and detailed magnifying colonoscopy findings. We examined the relation between these two subclasses and histology/invasion depth.

RESULTS: One hundred and four lesions (51.5%) were judged to be mildly irregular, and 98 lesions (48.5%) were judged to be severely irregular. Ninety-seven (93.3%) mildly irregular lesions showed dysplasias or submucosal invasion of less than 1000 μm (SM < 1000 μm). Fifty-five (56.1%) severely irregular lesions showed submucosal invasion equal to or deeper than 1000 μm (SM ⩾ 1000 μm). Mild irregularity was found significantly more often in dysplasias or lesions with SM < 1000 μm than in lesions with SM ⩾ 1000 μm ($P < 0.01$).

CONCLUSION: Subclassification of the type VI pit pattern is useful for identifying dysplasias or lesions with SM < 1000 μm.

INTRODUCTION

Pit pattern classification (Figure 1) of colorectal lesions, initially proposed by Kudo[1] and modified by Kudo and Tsuruta[2], is reported to be related to the histologic characteristics of the lesions[3-9]. Magnifying colonoscopy is used for differential diagnosis between non-neoplastic and neoplastic lesions[5,9-16] and for assessing the depth of invasion of early colorectal carcinoma[9,17-21].

Several studies have suggested that there is little risk of lymph node metastasis from early colorectal carcinoma that involves the superficial layer of the submucosa, less than 1000 μm from the muscularis mucosae[9,17,22-24]. Recently, the Japanese Society for Cancer of the Colon and Rectum proposed the following new criteria for curative histopathologic conditions after complete endoscopic mucosal resection (EMR) of submucosal carcinoma: (1) a submucosal invasion depth of less than 1000 μm (SM < 1000 μm), (2) well to moderately differentiated adenocarcinoma including the invasive portion, and (3) no vessel involvement[22,24]. In accordance with these proposed criteria, it has become important to distinguish submucosal invasion equal to or deeper than 1000 μm (SM ⩾ 1000 μm) from SM < 1000 μm prior to treatment of submucosal carcinoma, to reduce the number of needless surgical resections.

Many studies have shown that the type VN pit pattern is an indicator of massive submucosal invasion of colorectal neoplasm[9]. However, colorectal neoplasms with a type VI pit pattern include various lesions, such as dysplasia and submucosal carcinoma, with either SM < 1000 μm

or SM ⩾ 1000 μm[9]. Thus, it is difficult to decide upon a therapeutic strategy for colorectal neoplasm on the basis of the current type VI pit pattern classification. In this study, we assessed the clinical usefulness of type VI pit pattern subclassification in determining the histology/invasion depth of colorectal neoplasms.

MATERIALS AND METHODS

We analyzed 272 colorectal neoplasms with a type V pit pattern (type VI, n = 202; type VN, n = 70). The colorectal neoplasms comprised 117 dysplasias and 155 submucosal invasive carcinomas, 129 of which were deeper than 1000 μm, resected endoscopically or surgically from 228 patients at Hiroshima University Hospital during the period January 1999 through March 2005. All lesions in this study were analyzed by five colonoscopists who were well trained in magnifying colonoscopy and blinded to the pathology findings, retrospectively.

To evaluate pit patterns, we used a magnifying colonoscope (EC-410CM, EC-450ZM, EC-450ZH or EC-450ZW, Fujinon Toshiba, Omiya, Japan; or CF-240Z or CF-H260AZI, Olympus, Tokyo, Japan) with zoom functions ranging from × 17 to × 126. When a lesion was detected by standard colonoscopic observation, the surface mucus was washed away with lukewarm water, and indigo carmine dye was spread over the lesion. This dye enhances the colonoscopic appearance because it is retained within the pits and grooves of the mucosal surface. For more precise assessment, crystal violet stain was applied to the margins of the pits, rendering each pit pattern clearly visible in all cases. The type V pit pattern was classified as one of two subtypes according to the Kudo and Tsuruta classification system[1,2] (Figure 1): type VI, irregularly arranged and similar to type IIIL, IIIS, or IV patterns in size; and type VN, an area of obvious non-structure (as per the Hakone consensus meeting in April 2004)[9,25].

Each resected neoplasm was fixed routinely with 10% buffered formalin and embedded in paraffin, after which the entire tumor was cut into serial 2- to 3-mm thick slices. Microscopic examination of hematoxylin and eosin-stained sections was performed by one pathologist unaware of other features of the case. Dysplasia was defined according to the Vienna criteria[26]. According to previously proposed measuring methods[22,24], the depth of submucosal invasion was determined using a micrometer under a microscope, and taken as the distance from the muscularis mucosae to the point of the deepest invasion (tumor apex).

We first analyzed the relation between the type V pit pattern subtype (VI or VN) and histology/invasion depth. We then examined the relation between SM ⩾ 1000 μm and histology/invasion depth and the following five detailed magnifying colonoscopy findings: (1) irregularity of the pit margins, (2) staining characteristics of the areas between pits, (3) area diameter of the type VI pit pattern (< 5 mm or ⩾ 5 mm), (4) density of the pits, and (5) width of the intervening membrane between the pits (Figure 2).

We divided the lesions with a type VI pit pattern into two subclasses (mildly irregular lesions and severely irregular lesions) according to the prominent and detailed magnifying colonoscopy findings of the first analysis. Mildly irregular lesions were defined as lesions with one or no significant magnifying colonoscopy findings, and severely irregular lesions were defined as lesions with two or more significant magnifying colonoscopy findings. This was done to diagnose SM ⩾ 1000 μm on the basis of cluster analysis[27,28]. Using these data, we examined the relation between the type VI pit pattern subclassifications and histology/invasion depth, the status of the muscularis mucosae, and the presence of desmoplastic reactions at the surface of the lesion (Figure 3). The muscularis mucosae were classified as detected, partially disappeared, or disappeared, as reported previously[29]. Desmoplastic reaction of the submucosal layer was classified as absent (-), mild to moderate (+), or severe (++), as reported previously[18,30].

The associations of dysplasia, SM < 1000 μm, and SM ⩾ 1000 μm with the type V pit pattern subtypes, detailed magnifying colonoscopy findings, and the type VI lesion subclasses, were analyzed by chi-square test. $P < 0.05$ was accepted as statistically significant. In addition, to identify predictors of SM ⩾ 1000 μm, we performed multivariate logistic regression analysis. All statistical analyses were performed using JMP statistical software, version 5.0.1 J (SAS Institute Inc, Cary, NC).

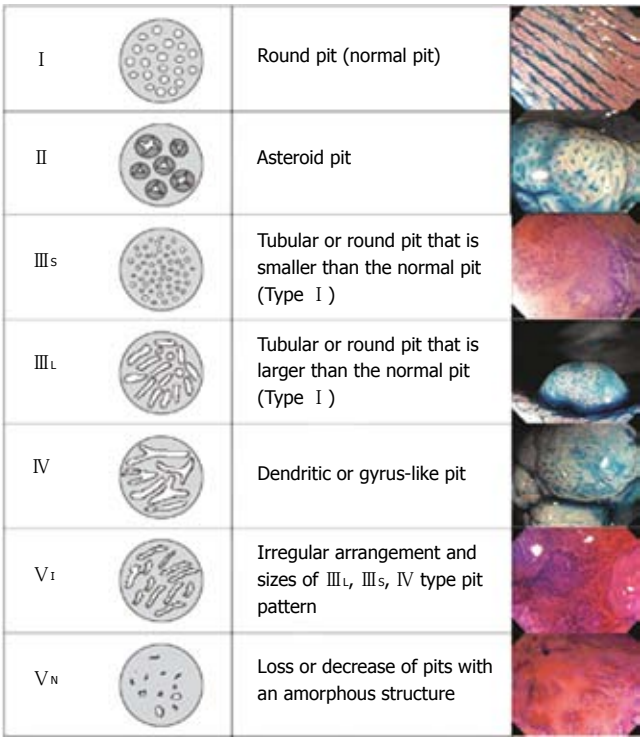


Figure 1 Classification of pit patterns of colorectal lesions.

RESULTS

Histology/invasion depth of colorectal neoplasm in relation to type V pit pattern subtypes

Dysplasia, SM < 1000 μm and SM ⩾ 1000 μm were found

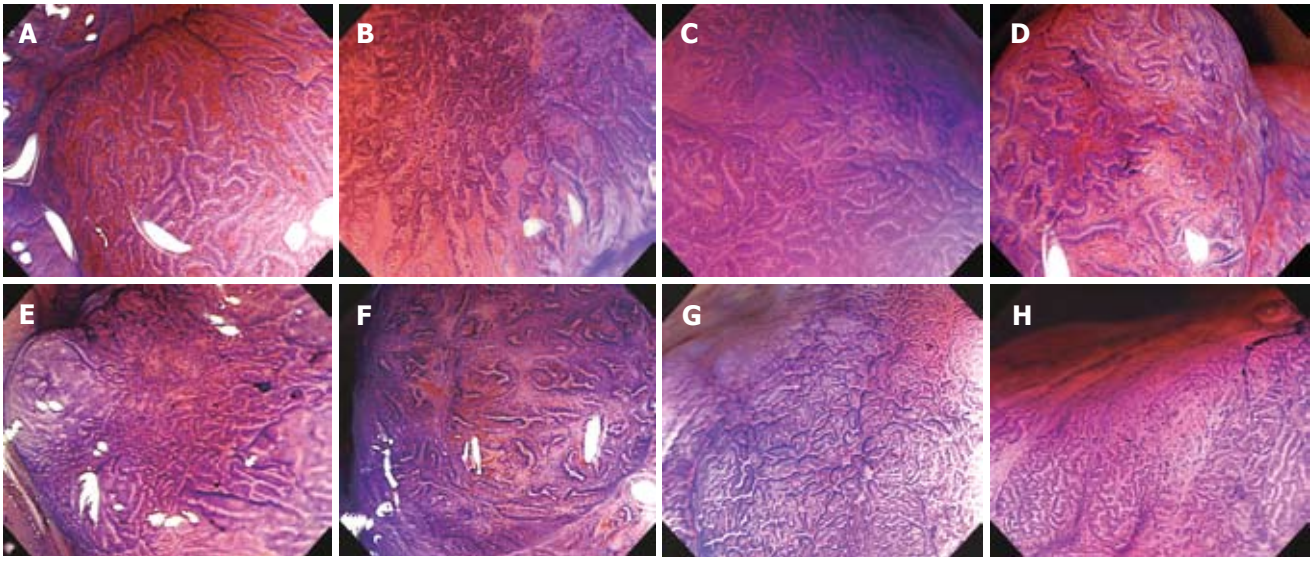


Figure 2 Magnifying features of colorectal neoplasm (crystal violet): **A**: Regular pit margins; **B**: Irregular pit margins; **C**: Clear staining characteristics of the areas between pits; **D**: Unclear staining characteristics of the areas between pits; **E**: High residual pit density; **F**: Low residual pit density; **G**: Narrow intervening membrane between pits; **H**: Wide intervening membrane between pits.

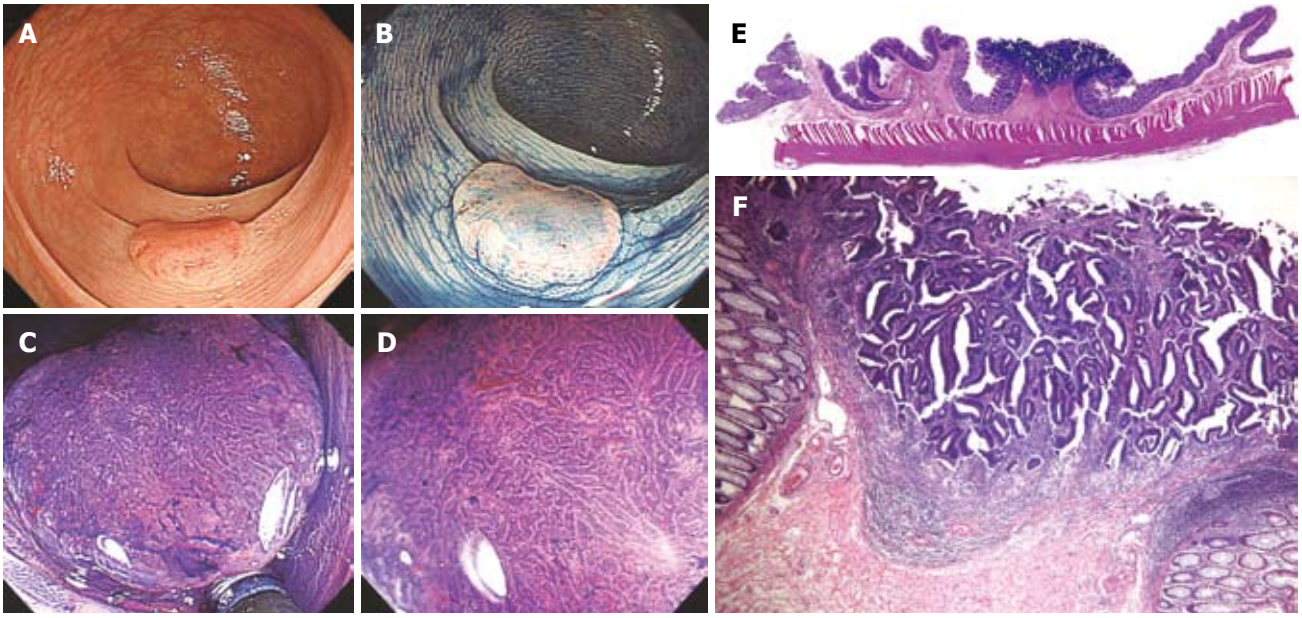


Figure 3 Type Ⅱa + 10Ⅱc lesion, 12 mm in diameter. **A**: Standard colonoscopic view; **B**: Standard colonoscopic view with indigo carmine spraying; **C**, **D**: Magnifying colonoscopic picture with crystal violet staining reveals type V_I pit pattern. Irregular pit margins, unclear staining characteristics of the areas between pits, > 5 mm area of type V_I pit pattern, high residual pit density, and narrow intervening membrane between pits is revealed; **E**: Cross-section (hematoxylin-eosin, × 8) of a surgically resected specimen showing submucosal invasion (1800 μm); **F**: Low-power view (hematoxylin-eosin, × 40) of type V_I pit pattern. Muscularis mucosae have disappeared. Desmoplastic reactions are mild to moderate. In this case, lymph node metastasis was not detected.

in association with 57.9% (117/202), 11.4% (23/202), and 30.7% (62/202) of the neoplasms with type V_I pit patterns, respectively (Table 1). Dysplasia, SM < 1000 μm, and SM ≥ 1000 μm were found in association with 0% (0/70), 4.3% (3/70), and 95.7% (67/70) of the neoplasms with type V_N pit patterns, respectively. The type V_N pit pattern was found significantly more often in lesions with SM ≥ 1000 than in dysplasias or lesions with SM < 1000 μm ($P < 0.01$). Sensitivity and specificity of the type V_N pit pattern for a diagnosis of SM ≥ 1000 were 51.9% (67/129) and 97.9% (140/143), respectively.

Histology/invasion depth of colorectal neoplasm with a type V_I pit pattern in relation to detailed magnifying colonoscopy findings

SM ≥ 1000 μm was found in association with 58.2% (46/79) of the lesions with irregular pit margins, 57.5% (50/87) of the lesions with unclear staining characteristics of the areas between pits, 41.3% (57/138) of the lesions with a type V_I pit pattern area ≥ 5 mm in diameter, 26.6% (29/109) of the lesions with high residual pit density, and 31.9% (44/138) of the lesions with a wide intervening membrane between pits (Table 2). SM ≥ 1000 μm was

Table 1 Histology/invasion depth of colorectal neoplasm in relation to type V pit pattern subtypes, *n* (%)

Type V pit pattern subtypes		Histology/invasion depth		
		Dysplasia	SM < 1000 μm	1000 μm ≤ SM
V_I	202 (100)	117 (57.9)	23 (11.4)	62 (30.7)
V_N[1]	70 (100)	0 (0)	3 (4.3)	67 (95.7)

[1]Dysplasia. SM < 1000 μm *vs* SM ≥ 1000 μm, $P < 0.01$. SM: Submucosa.

Table 2 Histology/invasion depth of colorectal neoplasm with type V_I pit pattern in relation to detailed magnifying colonoscopy findings, *n* (%)

Magnifying colonoscopy finding		Histology/invasion depth		
		Dysplasia	SM < 1000 μm	1000 μm ≤ SM
Irregular pit margins[1]	79 (100)	23 (29.1)	10 (12.7)	46 (58.2)
Unclear staining characteristics of the area between pits[1]	87 (100)	21 (24.1)	16 (18.4)	50 (57.5)
Area diameter of type V_I pit pattern ≥ 5 mm[1]	138 (100)	65 (47.1)	16 (11.6)	57 (41.3)
High residual pit density	109 (100)	64 (58.7)	16 (14.7)	29 (26.6)
Wide intervening membrane between pits	138 (100)	81 (58.7)	13 (9.4)	44 (31.9)

[1]Dysplasia. SM < 1000 μm *vs* SM ≥ 1000 μm, $P < 0.01$. SM: Submucosa.

found significantly more often in association with irregular pit margins, unclear staining characteristics of the areas between pits, and a type V_I pit pattern area ≥ 5 mm in diameter than in association with regular pit margins, clear staining characteristics of the areas between pits, and a type V_I pit pattern area < 5 mm in diameter.

Results of multivariate logistic regression analysis for predictors of SM ≥ 1000 μm

In multivariate logistic regression analysis, unclear staining characteristics of the areas between pits, irregular pit margins, and a V_I pit pattern area diameter of ≥ 5 mm were shown to be significant predictors of SM ≥ 1000 μm (Table 3). High residual pit density and a wide intervening membrane between pits were not significant.

Histology/invasion depth of colorectal neoplasm in relation to type V_I pit pattern subclassifications

One hundred and four lesions (51.5%) were judged to be mildly irregular, and 98 lesions (48.5%) were judged to be severely irregular (Table 4). Ninety-seven (93.3%) mildly irregular lesions showed dysplasias or SM < 1000 μm. Fifty-five (56.1%) severely irregular lesions showed SM ≥ 1000 μm. Mild irregularity was found significantly more often in dysplasias or in lesions with SM < 1000 μm than in lesions with SM ≥ 1000 ($P < 0.01$). Sensitivity and specificity of mild irregularity for dysplasias or SM < 1000 μm were 69.3% (97/140) and 88.7% (55/62), respectively.

Table 3 Results of multivariate logistic regression analysis for predictors of submucosal invasion deeper than 1000 μm (*n* = 202)

Magnifying colonoscopy finding	Odds ratio (*P* value)	Relevant finding
Unclear staining characteristics of the areas between pits	6.24 (< 0.0001)	Clear staining characteristics of the areas between pits
Irregular pit margins	4.89 (< 0.0001)	Regular pit margins
Area diameter of type V_I pit pattern ≥ 5 mm	4.14 (0.0132)	Area diameter of type V_I pit pattern < 5 mm
High residual pit density	1.51 (0.3335)	Low residual pit density
Wide intervening membrane between pits	1.02 (0.9740)	Narrow intervening membrane between pits

Table 4 Histology/invasion depth of colorectal neoplasm in relation to type V_I pit pattern subclassifications, *n* (%)

Type V_I pit pattern subclassification		Histology/invasion depth		
		Dysplasia	SM < 1000 μm	1000 μm ≤ SM
Mildly irregular[1]	104 (100)	89 (85.6)	8 (7.7)	7 (6.7)
Severely irregular	98 (100)	28 (28.6)	15 (15.3)	55 (56.1)

[1]Dysplasia. SM < 1000 μm *vs* SM ≥ 1000 μm, $P < 0.01$. SM: Submucosa.

Table 5 Status of muscularis mucosae in relation to type V pit patterns, *n* (%)

Type V pit pattern		Status of muscularis mucosae		
		Detected	Partially disappeared	Disappeared
V_I				
Mildly irregular[1]	104 (100)	97 (93.2)	6 (5.8)	1 (1.0)
Severely irregular[a]	98 (100)	38 (38.8)	31 (31.6)	29 (29.6)
V_N[b]	67 (100)		8 (11.9)	59 (88.1)

[1]Detected *vs* partially disappeared, disappeared, [b]$P < 0.01$, [a]$P < 0.05$.

Status of the muscularis mucosae in relation to type V pit patterns

The muscularis mucosae was detected in 97 (93.2%) mildly irregular lesions (Table 5). Partial disappearance or disappearance of the muscularis mucosae was seen in 60 (61.2%) severely irregular lesions and 67 (100%) lesions with a type V_N pit pattern. Severe irregularity was found significantly more often in association with partial disappearance or disappearance of the muscularis mucosae than in association with detection of the muscularis mucosae ($P < 0.05$). The type V_N pit pattern was found significantly more often in association with partial disappearance or disappearance of the muscularis mucosae than in association with detection of the muscularis mucosae ($P < 0.01$).

Desmoplastic reactions at the surface of the lesion in relation to type V pit patterns

No desmoplastic reaction of the superficial layer was observed in 100 (96.2%) mildly irregular lesions (Table 6). Desmoplastic reactions (+)/(++) were observed in 50

(51.0%) severely irregular lesions and 67 (100%) lesions with a type VN pit pattern. The type VN pit pattern was found significantly more often in lesions with a desmoplastic reaction (+)/(++) than in lesions with desmoplastic reaction (-) ($P < 0.01$).

DISCUSSION

Endoscopic treatment, such as EMR, is both a therapeutic technique and an important diagnostic technique. Therefore, it is important to be able to identify lesions for which endoscopic resection would be curative to avoid meaningless endoscopic resection for lesions that should be treated surgically. Pit pattern classification is used clinically to help determine the best treatment for colorectal tumors[9]. Type Ⅰ and Ⅱ pit patterns predict nonneoplastic lesions, whereas type Ⅲ, Ⅳ, and Ⅴ pit patterns predict neoplastic lesions. Lesions with a type Ⅲ or Ⅳ pit pattern are almost always dysplasias and are thus indications for endoscopic resection. Almost all lesions with a type VN pit pattern show SM ⩾ 1000 μm. The reported accuracy of detection of massive submucosal invasion on the basis of the type VN pit pattern is 97%[23]. In our study, SM ⩾ 1000 μm was found in 95.7% of lesions with a type VN pit pattern. Therefore, surgical resection is indicated for such lesions. By contrast, lesions with a type VI pit pattern include dysplasia and various submucosal carcinomas; thus, it is difficult to decide upon a therapeutic strategy on the basis of the current pit pattern classification system. It is necessary to analyze the type VI pit pattern in detail to determine the appropriate therapeutic strategy. The present study revealed that irregular pit margins, unclear staining characteristics of the areas between pits, and a type VI pit pattern area diameter ⩾ 5 mm are significant predictors for submucosal invasion of colorectal neoplasms of 1000 μm or more.

Lesions that were subclassified as mildly irregular lesions were mainly dysplasias or lesions that showed SM < 1000 μm (93.3%). Therefore, endoscopic resection is indicated for mildly irregular lesions. On the contrary, lesions that were classified as severely irregular lesions included not only dysplasias or lesions with SM < 1000 μm (43.9%), but also lesions with SM ⩾ 1000 μm (56.1%). For severely irregular lesions, the therapeutic strategy should be determined on the basis of standard endoscopic findings in conjunction with those of other modalities, such as contrast enema radiography or endoscopic ultrasonography[29,31,32]. New diagnostic modalities, such as narrow band imaging, are expected to provide more information about the invasion depth of colorectal carcinomas[33-36].

Our results revealed that there is a significant histologic difference between mildly irregular lesions and severely irregular lesions. The degree of disappearance of the muscularis mucosae increased as the pit patterns changed from VI with mildly irregularity to VI with severely irregularity to VN. If we could determine the status of the muscularis mucosae by magnifying colonoscopy, the pit pattern would be helpful in determining the depth of submucosal invasion depth by endoscopic ultrasonography. The muscularis mucosae had disappeared in all lesions with a type VN pit pattern; thus, we can measure the invasion depth from the surface of a lesion of this type to the deepest portion[29]. It has been reported that desmoplastic reactions are related to massive submucosal invasion[18]. In the present study, the incidence of desmoplastic reactions increased as the pit patterns changed from VI with mildly irregularity to VI with severely irregularity to VN. There were no desmoplastic reactions in mildly irregular lesions. These results indicate that changes in the appearance of the pits are caused by the process of submucosal infiltration of the colorectal neoplasm. Although the mechanism underlying this process is not clear, it is possible that irregular pit margins and unclear staining characteristics of the areas between pits may involve several molecular markers. We reported previously that the proliferation, infiltration and lymph node metastasis of submucosal colorectal carcinoma are significantly related to the expression of markers such as Ki-67, E-cadherins, MUC1, cathepsin D and MMP-7 at the deepest portion[37-43]. We also reported previously that MUC1 expression at the superficial layer may be related to colorectal tumors with a type V pit pattern[42]. However, there are few reports pertaining to the relation between the expression of specific molecular markers and morphogenesis of the type VI pit pattern. There may be a relation between the expression of molecular markers and detailed magnifying colonoscopy features of the type VI pit pattern. Further investigation will clarify the relation between molecular morphogenesis at the lesion surface and type VI pit pattern subclassifications.

We conclude that type VI pit pattern subclassification is useful for identifying dysplasias or lesions with SM < 1000 μm. Subclassifications can be applied to decisions about whether endoscopic treatment is indicated for colorectal neoplasms. However, we cannot identify lesions with SM ⩾ 1000 μm on the basis of type VI pit pattern subclassifications.

Table 6 Desmoplastic reaction at the lesion surface in relation to type V pit pattern, *n* (%)

Type V pit pattern		Desmoplastic reaction		
		Absent (-)	Mild to moderate (+)	Severe (++)
VI				
Mildly irregular	104 (100)	100 (96.2)	4 (3.8)	
Severely irregular	98 (100)	48 (49.0)	29 (29.6)	21 (21.4)
VN[b]	67 (100)		16 (23.9)	51 (76.1)

[b]$P < 0.01$ (-) *vs* (+), (++).

COMMENTS

Background

Colorectal neoplasms with a type VI pit pattern include various lesions, such as dysplasias and submucosal carcinomas, with either SM < 1000 μm or SM ⩾ 1000 μm. Thus, it is difficult to decide upon a therapeutic strategy for colorectal neoplasm on the basis of the current type VI pit pattern classification.

Research frontiers

In this study, we assessed the clinical usefulness of type VI pit pattern subclassification in determining the histology/invasion depth of colorectal neoplasms. There has been little study on type VI pit pattern subclassification.

Innovations and breakthroughs

Type V_I pit pattern subclassification is useful for identifying dysplasias or lesions with SM < 1000 μm.

Applications

Subclassifications can be applied to deciding whether endoscopic treatment is indicated for colorectal neoplasms.

Terminology

Type V_I pit pattern subclassification: We divided the lesions with a type V_I pit pattern into two subclasses (mildly irregular lesions and severely irregular lesions) according to the prominent detailed magnifying colonoscopy findings of the first analysis. Mildly irregular lesions were defined as lesions with one or no significant magnifying colonoscopy findings, and severely irregular lesions were defined as lesions with two or more significant magnifying colonoscopy findings.

Peer review

The authors retrospectively investigated whether subclassification of the type V_I pit pattern on the basis of magnifying colonoscopy findings was useful in determining the type and depth of invasion of colorectal neoplasm. They concluded that subclassification of the type V_I pit pattern is useful for identifying dysplasias or lesions with SM < 1000 μm.

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RAPID COMMUNICATION

Detection of hMSH2 and hMLH1 mutations in Chinese hereditary non-polyposis colorectal cancer kindreds

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Abstract

AIM: To establish and validate the mutation testing for identification and characterization of hereditary non-polyposis colorectal cancer (HNPCC) in suspected Chinese patients.

METHODS: Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. Genomic DNA was extracted after informed consent was obtained. The coding region of hMSH2 and hMLH1 genes was detected by polymerase chain reaction (PCR) and denaturing high-performance liquid chromatography (DHPLC). Mutations identified in the proband by DHPLC were directly sequenced using a 377 DNA sequencer, analyzed with a basic local alignment tool (BLAST), and tested in the corresponding family members by direct DNA sequencing.

RESULTS: Mutations were identified in two Chinese HNPCC kindreds. One was the missense mutation of hMSH2 c.1808A→G resulting in Asp 603 Gly identified in the proband of the fifth HNPCC (HNPCC5) kindred. In the HNP5 kindred, three family members were found to have this mutation and two of them had colorectal cancer. The other mutation of hMLH1 c.1882A→G was identified in the HNP2 kindred's proband, which might be the nonsense mutation analyzed by BLAST.

CONCLUSION: Pedigree investigation and mutation testing of hMSH2 and hMLH1 are the practical methods to identify high-risk HNPCC patients in China.

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INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) syndrome is characterized by autosome-dominantly inherited predisposition to early colorectal carcinoma and extracolonic epithelia-derived tumors most often located in the gastrointestinal and urogenital tracts[1]. The mean age of HNPCC and sporadic colorectal cancer (CRC) patients at diagnosis is 42 years and 65 years, respectively. HNPCC, accounting for approximately 5%-15% of all CRCs, is categorized as Lynch I or Lynch Ⅱ syndrome (according to revised Amsterdam criteria)[2]. Germline mutations of the mismatch repair (MMR) genes identified in HNPCC kindreds, including *MSH2*, *MLH1*, *MSH6*, *PMS1* (promotion of mutS homology 1), *PMS2*, and *MLH3*, have been proved as the major cause for HNPCC by linkage analysis. Mutations in two of these MMR genes, *MSH2* and *MLH1*, account for the majority of the kindreds with HNPCC[3]. Thus, pedigree investigation and MMR gene testing, as the basis for efficient prevention and treatment of HNPCC, are most often used in early diagnosis of at-risk family members and in confirmation of the diagnosis of HNPCC.

CRC is the third life-threatening cancer in China. However, HNPCC pedigree and its predisposition gene have not been extensively studied. We have established a CRC database since 1994 and the follow-up rate is above 90%. We have recently paid attention to hereditary colorectal cancer in South China and found that about 3% of CRC patients have multiple CRCs to which the young are vulnerable[4,5]. Thus, we collected those CRC families and finally obtained eleven independent Chinese kindreds with HNPCC by deep pedigree investigation until January,

2004. Five of them fulfilled the classical Amsterdam criteria. To identify high-risk populations with HNPCC, we tested hMSH2 and hMLH1 mutations in these classical kindreds.

MATERIALS AND METHODS

Patients

The clinical diagnosis of classical HNPCC was established and verified at the Department of Gastrointestinopan creatic surgery, the First Affiliated Hospital of the Sun Yat-Sen University[2]. Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. The study was approved by the Ethical Committee of Sun Yat-Sen University.

DNA isolation

Peripheral blood samples were collected from both patients and their family members in each Chinese kindred after informed consent was obtained for genetic analysis. DNA was extracted directly from leukocytes following the standard procedures.

All exons of *MLH1* and *MSH2* were amplified for mutation testing. Samples used were amplified in 20 mL reaction volume containing approximately 100 ng DNA in 50 mmol/L KCl, 10 mmol/L Tris-HCl, 1.5 mmol/L $MgCl_2$, 200 mmol/L each dNTP, 0.01% gelatin, 1 U Taq-polymerase, and 20-40 pmol each primer. The PCR amplification conditions were as follows: denaturation at 95℃ for 5 min, followed by 30 cycles of denaturation at 95℃ for 30 s, annealing at fragment-specific annealing temperature for 1 min, extension at 72℃ for 1 min, and a final extension at 72℃ for 7 min. The primers, annealing temperature, and size of the PCR products for each of the investigated hMLH1 and hMSH2 exons are described elsewhere[6-8]. The primers were synthesized by Shanghai Bio-Chemical Corporation.

DHPLC analysis

The amplified PCR fragments were screened for sequence variants by denaturing high pressure liquid chromatography (DHPLC) on a WAVE DNA fragment analysis system (Transgenomic, San Jose, CA, USA). The running conditions for each amplicon (available upon request) were determined by the Wavemaker 3.4.4 software (Transgenomic, San Jose, CA, USA) based on the DNA sequence. Five mL of each PCR product (containing 50-100 ng of DNA) was denaturated at 95℃ for 3 min and then gradually reannealed by decreasing the sample temperature from 95℃ to 65℃ over 30 min. PCR products were then separated at a flow rate of 0.9 mL/min with a linear acetonitrile gradient. Generally, analysis took approximately 10 min including column regeneration and re-equilibration to starting conditions. The column mobile phase consisted of a mixture of 0.1 mol/L triethylamine acetate (pH 7.0) with (buffer B) or without (buffer A) 25% acetonitrile. Samples displaying variant elution peaks in each run were chosen for sequence analysis. For some amplicons displaying variations in elution profiles of control samples, all samples were sequenced.

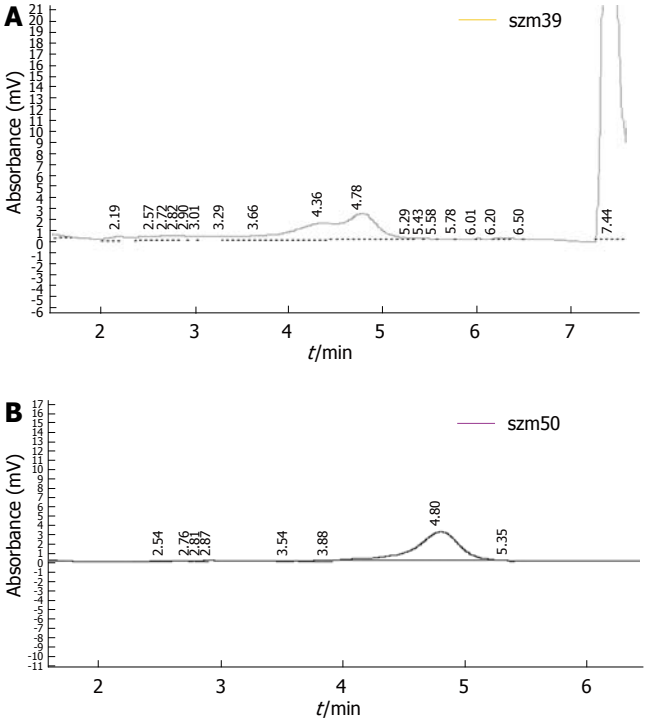


Figure 1 DHPLC showing variant elution peaks (**A**) and normal elution peaks (**B**) in exon 12 of hMSH2 gene.

DNA sequencing

DHPLC variants were confirmed by direct sequencing of independently amplified PCR products of the amplicons, in both sense and antisense direction, using the same primers. Sequencing was performed with Big Dye™ terminator cycle sequencing ready reaction kit (Applied Biosystems, Inc., Foster City, CA) on an ABI Prism™ 377 DNA sequencer following the standard conditions recommended by the manufacturer. Sequences obtained were aligned and compared to published wild-type sequences by Sequencher 3.1.1 analysis software.

Mutations identified in the kindred's proband were tested among their family members by direct DNA sequencing.

RESULTS

Among the 5 probands of the five Chinese HNPCC kindreds, 180 PCR products were screened by DHPLC. Two PCR products found with variant elution peaks by DHPLC were identified to have mutations. One was hMSH2 c.1808A→G resulting in Asp 603 Gly identified in the HNP5 kindred's proband and tested among the family members (Figures 1-3). The other was hMLH1 c.1882A →G identified in the HNP2 kindred's proband (Figures 4 and 5), which was not tested among the family members, because it might be the nonsense mutation analyzed by BLAST.

As shown in Figure 3, in the HNPCC5 kindred, there were four colorectal carcinoma patients in two successive generations, and three of these were diagnosed before the age of 45 years. The proband developed endometrial carcinoma at the age of 61 years, bladder carcinoma at the age of 66 years and colorectal carcinoma at the age of 72

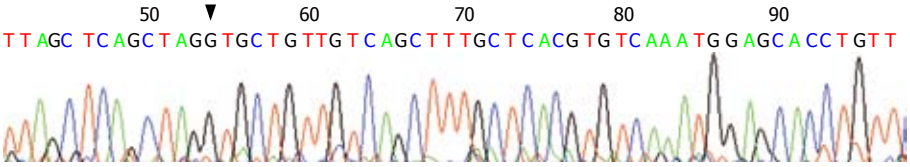


Figure 2 HPLC variants confirmed by direct sequencing displaying a single nucleotide substitution of c.1808A→G in exon 12 of hMSH2 gene.

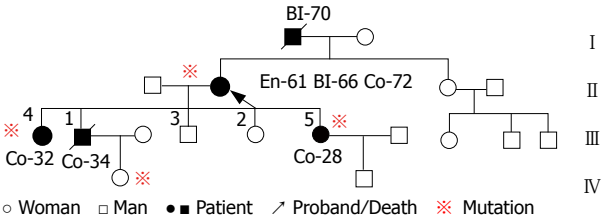


Figure 3 Pedigree tree of HNPCC 5 kindred. Co: Colon; Bl: Bladder; En: Endometrium.

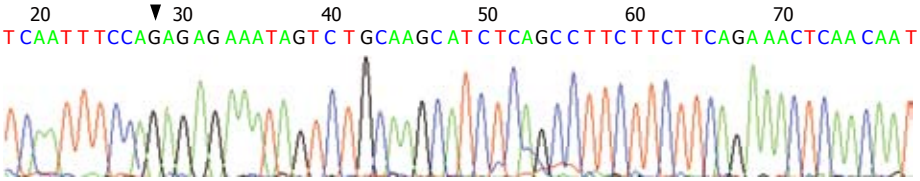


Figure 4 DHPLC variants confirmed by direct sequencing revealing a single nucleotide substitution of c.1882A→G in exon 16 of hMLH1 gene.

years, while his father developed bladder carcinoma at the age of 70 years. In addition, patient Ⅲ-1 had colorectal carcinoma at the age of 34 years and died of synchronous hepatic metastasis.

In the HNP5 kindred, each PCR product from nine proband's family members was tested by direct DNA sequencing. Only three of them were found to have the missense mutation in hMSH2 at position A1808G. The missense mutation sequence variant was found in exon 12 of hMSH2 gene. It was a single nucleotide substitution of c.1808A→G (Figure 2), which resulted in Asp 603 Gly of hMSH2 (NCBI Ref. Seq. NM 000251 and NP 000242 for mRNA and protein, respectively). Proband and patient Ⅲ-4 and -5 had this mutation and developed colorectal carcinoma. Patient Ⅲ-1's daughter had no colorectal disease even though she had this mutation, and was still under follow-up. No mutation was found in the others.

DISCUSSION

CRC is one of the most common cancers and its clinical selection criteria for cancer families were first established in Amsterdam in 1990 by the International Collaborative Group on HNPCC and modified in 1999[2]. Detection of constitutional mutations in genes associated with predisposition to cancer is practical in molecular diagnosis. Data suggest that molecular testing is much more efficient, if analyses are focused on a limited number of alterations[9-13]. Detection of germline mutation carriers is an efficient method to define high-risk CRC patients. Identification of germline mutations of either hMSH2 or hMLH1 could be performed in 50%-70% of families meeting the Amsterdam criteria for HNPCC, whereas the families not complying with these criteria show a much lower frequency of the MMR gene mutations[14-17]. Thus, hMSH2 or hMLH1 gene testing is most often used in early diagnosis of at-risk family members with HNPCC.

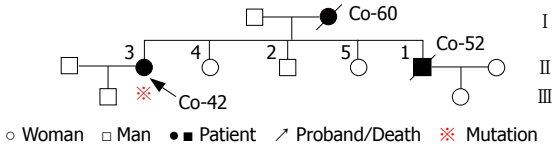


Figure 5 Pedigree tree of HNPCC 2 kindred. Co: Colon.

To identify a population-specific panel of mutations, it is crucial to describe them in all ethnic groups.

Even though CRC is common in China, few studies on HNPCC pedigree and its predisposition gene are available[4,17]. In our study, HNPCC kindreds were collected and hMSH2 and hMLH1 gene mutations were tested in order to find high-risk CRC populations. Two mutations were found among the five Chinese HNPCC kindreds. In the 5HNPCC kindreds, missense mutation was found in four members, three of them developed colorectal carcinoma. Although it has not been confirmed as a germline mutation yet, it may be an important factor for CRC development. Thus, the patient Ⅲ-1's daughter with this mutation should be closely followed up.

Approximately 20% of patients with colorectal cancer have a genetic component and HNPCC is the most common autosome dominant hereditary syndrome predisposing to colorectal cancer[18,19]. Various methods have been described to screen for HNPCC and directly test for mismatch repair gene mutations[20-27]. For patients with available tumor specimens, MSI and IHC are widely performed. Ruszkiewicz *et al*[28] reported that immunohistochemistry is an alternative method for assessment of MSI status, which is fast and relatively inexpensive compared with MSI testing. Some reports[26,29,30] suggest that detection of MSI and IHC for hMSH2/hMLH1 proteins is a reliable pre-screening test for hMLH1/hMSH2 germline mutations in families suspected of having HNPCC. Because China is a developing country with a large population and the incidence rate of CRC

increases, a screening protocol specific for the Chinese population is necessary to detect the high-risk populations with HNPCC. hMSH2 and hMLH1 gene mutation testing is a practical method for detecting HNPCC in Chinese population. Meanwhile, further research should be performed in Chinese HNPCC kindreds with germline mutations.

COMMENTS

Background

Approximately 20% of colorectal cancer patients have a genetic component and hereditary non-polyposis colorectal cancer (HNPCC) is the most common autosome dominant hereditary syndrome predisposing to colorectal cancer. Various methods have been described to screen for HNPCC and directly test for mismatch repair gene mutations. Even though CRC is common in China, few studies on HNPCC pedigree and its predisposition gene are available.

Research frontiers

Data suggest that molecular testing is much more efficient when analyses are focused on a limited number of alterations. Detection of germline mutation carriers is an efficient method to define high-risk CRC patients. Germline mutations of either hMSH2 or hMLH1 can be identified in 50%-70% of families meeting the Amsterdam criteria for HNPCC. Thus, hMSH2 or hMLH1 gene testing is most often used in early diagnosis of at-risk family members with HNPCC.

Innovations and breakthroughs

Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. Mutations were identified in two Chinese HNPCC kindreds. One was the missense mutation of hMSH2 c.1808A→G resulting in Asp 603 Gly, the other was the mutation of hMLH1 c.1882A→G .

Applications

Molecular pathological tests should be performed in order to identify individuals and at-risk family members with HNPCC. Close follow-up and intensive surveillance should be performed.

Terminology

Denaturing high-performance liquid chromatography (DHPLC) is a method for separating DNA duplexes that differ in the identity of one or more base pairs. The method is believed to be most efficient at the site of mutation. Basic local alignment search tool (BLAST) is powerful to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be obtained from well designed queries and alignments. BLAST 2.0 can rapidly search for nucleotide and protein in their databases.

Peer review

The results of this clinical study indicate that mutation testing for hMSH2 and hMLH1 by PCR and direct sequencing is a preferable method to identify high-risk HNPCC patients.

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BASIC RESEARCH

Expression of thymidine kinase mediated by a novel non-viral delivery system under the control of vascular endothelial growth factor receptor 2 promoter selectively kills human umbilical vein endothelial cells

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Abstract

AIM: To investigate the killing efficiency of a recombinant plasmid containing a thymidine kinase (TK) domain insert driven by the vascular endothelial growth factor receptor 2 (VEGFR2) promoter (KDR) on vascular endothelial cells.

METHODS: The KDR-TK fragment was extracted from pBluescript Ⅱ KDR-TK plasmid by enzymatic digestion with *Xho*I and *Sal*I. The enhanced green fluorescence protein (EGFP) carrier was extracted from pEGFP by the same procedure. The KDR-TK was inserted into the pEGFP carrier to construct pEGFP-KDR-TK. Using ultrasound irradiation and microbubble, pEGFP-KDR-TK was transferred into human umbilical vein endothelial cells (HUVECs). The transient infection rate was estimated by green fluorescent protein (GFP) expression. Transfected HUVECs, non-transfected HUVECs, and HepG2 cells were cultured in the presence of different concentrations of ganciclovir (GCV), and the killing efficacy of HSV-TK/GCV was analyzed by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay.

RESULTS: The recombinant pEGFP-KDR-TK was successfully constructed by inserting the KDR-TK fragment into the pEGFP carrier. Transfected HUVECs showed cytoplasmic green fluorescence, and the transient transfection rate was about 20.3%. Pools of G418-resistant cells exhibited a higher sensitivity to the prodrug/GCV compared to non-transfected HUVECs or non-transfected HepG2 cells, respectively.

CONCLUSION: KDR promoter and the suicide gene/prodrug system mediated by diagnostic ultrasound combined with microbubble can significantly kill HUVECs. Such therapy may present a novel and attractive approach to target gene therapy on tumor vessels.

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Key words: Microbubble; Ultrasound; Gene therapy; Vascular endothelial growth factor receptor 2; Human umbilical vein endothelial cells

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Wang Y, Xu HX, Lu MD, Tang Q. Expression of thymidine kinase mediated by a novel non-viral delivery system under the control of vascular endothelial growth factor receptor 2 promoter selectively kills human umbilical vein endothelial cells. *World J Gastroenterol* 2008; 14(2): 224-230

http://www.wjgnet.com/1007-9327/14/224.asp

INTRODUCTION

Angiogenesis coincides with increased tumor cell entry into the blood circulation and thus facilitates metastasis. Previous reports revealed that targeting angiogenesis represents a new strategy for antitumor therapy[1].

Vascular endothelial growth factor-A (VEGF-A) is the major player in tumor angiogenesis, which activates two tyrosine kinase receptors VEGFR1 (Flt-1) and VEGFR2 (KDR in humans/Flk-1 in mice). KDR was found to be overexpressed on activated endothelial cells of newly formed vessels and strongly associated with invasion and metastasis in human malignant diseases[2].

Gene therapy has made significant progress in tumor therapy[3]. The effectiveness of suicide gene strategy directed against cancer cells has been shown for various tumor cell types[4]. Among several suicide gene prodrug combinations, the herpes simplex virus-thymidine kinase (HSV-TK) gene is a prototype. However, non-selective, ubiquitous expression of suicide genes may

cause unexpected adverse effects such as bone marrow suppression[5,6]. Thus, prerequisite for achieving effective and safe gene therapy against cancer is the induction of a strong, tumor vascular-selective expression of suicide genes. The success of gene therapy largely depends on the development of a gene delivery vector that is safe, easy-performed, and efficient. Ultrasound exposure in combination with microbubble is a new non-viral delivery system that might improve transfection efficiency. Nie *et al*[7] have shown that a combination of microbubble contrast agents and ultrasound irradiation might be a beneficial and much easier way for non-viral gene therapy.

In the present study, we established a KDR promoter to drive TK gene expression in human umbilical vein endothelial cells (HUVECs). Transfection was achieved by diagnostic ultrasound combined with a microbubble contrast agent. Our purpose was to analyze the cytotoxic effect of TK/ganciclovir (GCV) under the control of the KDR promoter on HUVECs.

MATERIALS AND METHODS

Cell isolation and culture

Primary HUVECs were isolated using the modified "irrigative digestion" technique as described by Jaffe *et al*[8]. Briefly, a transfusion silica gel tube was inserted into one end of new born aseptic umbilical cords after abdominal delivery from healthy pregnant women who had normal routine urinalysis and blood tests (women with any complications of pregnancy or viral infections were excluded) to flush the bloodstain remaining in the cavity repeatedly with PBS, and then 15-20 mL collagenase Ⅱ (Sigma, St. Louis, MO, USA) was injected into the vein cavity and retained 10-12 min for digestion. The collection of umbilical vein was flushed with PBS and the endothelial cells were collected and centrifuged at 1000 rpm for 8 min. Cells were resuspended and seeded in human endothelial-serum free medium (SFM) (Gibco, California, USA) supplemented with 100 mL/L fetal bovine serum (FBS, Gibco), 2 ng/mL vascular endothelial cell growth factor (Sigma), 90 μg/mL heparin (Sigma), 100 U/mL penicillin, and 50 μg/mL streptomycin. Culture medium was changed after 24 h and then every three days (3 mL) till the cells became a confluent monolayer. The primary cells were allowed to grow for 3 passages (1:2 split). Viability was assessed by trypan blue dye exclusion staining (Sangong Biological Engineering technology, Shanghai, China). HUVECs were identified by staining with rabbit anti-human von Willebrand factor (Boshide Company, Wuhan, China), as well as by performing the acetylated low-density lipoprotein color reagent test (DiI-Ac-LDL) (Molecular Probes, Novato, Canada, Europe BV). Experiments were performed on cultures with a viability > 95%. The human hepatoma cell line HepG2 was obtained from Laboratory of Surgery of our institution and cultured in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% FBS. The level of KDR expression in HUVEC and HepG2 cells was determined immunohistochemically.

Preparation of plasmid DNA

The pBluescript Ⅱ KDR-TK plasmid, which contains Herpes simplex virus thymidine kinase (HSV-TK) and the restriction sites for *Xho*I and *Sal*I and which is driven by the KDR promoter, was kindly provided by Dr Li Baojin. The 2.2-kb KDR-TK fragment was extracted from pBluescript Ⅱ KDR-TK by a double digestion with *Xho*I and *Sal*I (Takara Biotechnology Co.,Ltd, Dalian, China) and identified by electrophoresis and DNA sequencing. The pEGFP 4.7 kb carrier was released from EGFP plasmid DNA (donated by Doctor Zhang Ge, Laboratory of Molecular Medicine, Sun Yat-Sen University, China) containing the restriction sites for *Xho*I and *Sal*I using the same technique. The KDR-TK was inserted into the same clone location of the pEGFP vector with T4 ligase (Takara) to construct a plasmid termed pEGFP-KDR-TK. Restriction enzyme digestion and DNA sequencing (Bioasia Biotech Ltd, Shanghai, China) were used to confirm correct construction. The plasmid EGFP-KDR-TK was propagated in *E. coli* strain DH5α and purified using a large-scale plasmid DNA purification kit (Plasmid Maxi Kit; Qiagen, Hilden, Germany). The purity of EGFP-KDR-TK plasmid DNA was assessed by ultraviolet spectrophotometry.

Preparation of microbubble contrast agent

The ultrasound contrast agent SonoVueTM (Bracco SpA, Milan, Italy) that was used in this study consists of 59 mg of sulfur hexafluoride gas (SF6) and 25 mg of a freeze-dried white powder. Reconstitution of the material with 5 mL saline, yielded phospholipidstabilized microbubbles filled with sulfur hexafluoride with a diameter of less than 8 μm (mean, 2.5 μm)[9].

Gene transfer into cells with microbubble-enhanced ultrasound irradiation

HUVECs were seeded in 24-well plates at a density of 1.5×10^5 cells/well together with 15.0 μg of pEGFP-KDR-TK (50 μg/mL) in a total volume of approximately 300 μL and incubated for 20 min at 37℃. The 24-well plates were randomly divided into three groups with 8 samples in each group: group A, sham-exposure to ultrasound without addition of SonoVue; group B, exposure to ultrasound without addition of SonoVue, and group C, exposure to ultrasound with the addition of 2% SonoVue (600 μg/well). The ultrasound machine used was HP-Viewpoint 2500 scanner (Phillips Medical System, Bothell, MA, USA). An E94K213 transcranial vector transducer probe (12 mm in diameter) was placed on a polyester film (thickness, 6 μm) that covered the 24-well plate with 10 mm thickened contact gel between the probe and the polyester film, and the cells were exposed to 1.9 MHz continuous ultrasound for 5 min at an 80.0 mW/cm^2 output intensity (Figure 1). Five minutes after the first exposure, another 3 min exposure was performed under the same conditions. Cell viability was tested immediately after exposure by trypan blue dye exclusion staining. Cells were then incubated in SFM, 10% FBS, and 2 ng/mL vascular endothelial cell growth factor in 5% CO_2 at 37℃ for 48 h. Transfection efficiency was determined by fluorescence microscopy (Leica, Danaher Corporation, Wetzlar, Germany).

Subsequently, the cells were incubated for another 4 h

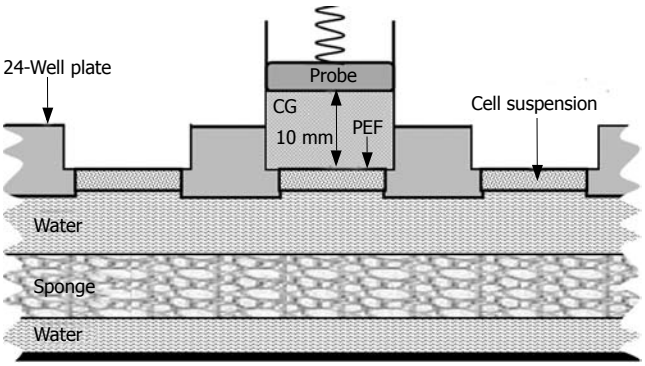


Figure 1 Schematic diagram of gene transfection. Transducer with contact gel (CG) was placed on the cell suspension covered with a 6-μm poly-ester film (PEF) under 1.9 MHz continuous ultrasound with a 80.0 mW/cm^2 output intensity. The 24-well plate was kept in a water bath at 37℃, and a sponge mat was placed in the water bath.

before they were subjected to selection with 600 μg/mL G418 (Gibco). At that time point when most of the cells in group A had died, G418 concentration was decreased to 200 μg/mL to maintain filtrate efficacy. After around three weeks of selection, a stable G418-resistant cell pool has formed as visualized by fluorescence microscopy. Cell colonies named KDR-TK/VEC were transferred with filter paper dipped trypsin to anther 24-well plate and cultured in an humidified atmosphere with 5% CO_2 at 37℃ for 3 d for subsequent experiments.

Sensitivity of cells to prodrug

KDR-TK /VEC, HUVEC, and HepG2 cells in logarithm growth term were detached by amylopsin (Sigma), the number of viable cells was determined by trypan blue dye exclusion, and viable cells were seeded in 96-well plates at a density of 2×10^3 cells/well. One day later, the cells were treated with 500 μL of fresh medium in the absence or presence of varying concentrations of GCV (0, 1, 10, 50, or 100 μg/mL). After incubation for 3 d, the sensitivity of cells to GCV was assessed using conventional 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay[10]. The percentage of suppressive cells was calculated with the following formula:

Relative suppressive rate = (1 - average OD570 of experiment/OD570 of control) × 100%.

Statistical analysis

Data were expressed as mean ± SD. The statistical significance of the data was compared by using two paired Student *t* test. ANOVA was used to analyze the differences among the different groups. Results were considered significant at $P < 0.05$.

RESULTS

Identification of HUVEC and KDR expression in cell lines

The average amounts of the endothelial cells isolated from umbilical cords using modified "irrigative digestion" technique were 1×10^6 cells. Light microscopic studies found that after 5 d in culture, HUVECs grew in confluent monolayer without a definable whirling pattern. The cells

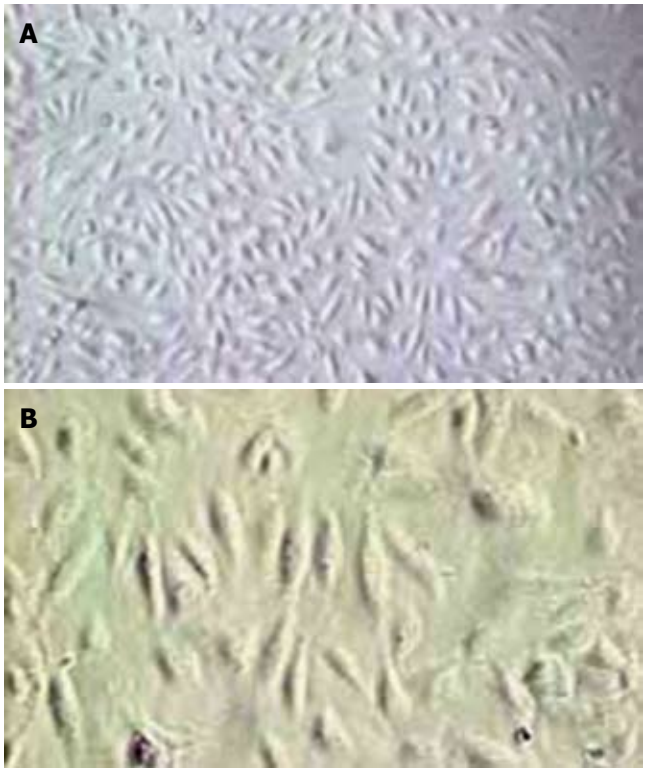


Figure 2 Photomicrographs show cultured human endothelial cells on d 7. The cells appeared to be a monolayer of very flat, polygonal-shaped cells. **A**: × 100; **B**: × 200.

were homogenous, closely opposed, large, and polygonal with an oval centrally coated nucleus and indistinct cell border (Figure 2). Factor Ⅷ is an important plasma marker of endothelium damage/dysfunction, which can also be detected in death or dysfunctional endothelial cells. The positive dye was brown in cytoplasm of HUVEC stained with the factor Ⅷ related antigen (Figure 3A). Compared with the factor Ⅷ, DiI-Ac-LDL can be taken up only by the living endothelial cells. The living HUVECs incubated with DiI-Ac-LDL are found to be red fluorescence under the fluorescent photomicroscope (Figure 3B), while the dead cells are uncolored. In the present study, the DiI-Ac-LDL stain revealed that more than 95% HUVEC were alive. To estimate the selectivity of the strategy using the KDR promoter, it is necessary to identify KDR-positive and KDR-negative cell lines. As shown in Figure 4, immunohistochemical analysis revealed KDR expression in HUVECs but not in HepG2.

Identification of recombinant plasmids

The positive clone of recombinant pEGFP-KDR-TK, which contains the neomycin resistance gene and the independent KDR promoters controlling the expression of TK, was identified by restriction endonuclease cleavage and sequencing. DNA sequencing and electrophoresis showed correct KDR-TK insertion into the pEGFP carrier (Figure 5).

Transfection of HUVECs with pEGFP-KDR-TK

The transient transfection rate was assessed by fluorescence microscopy 48 h after transfection. Fluorescent microscopy

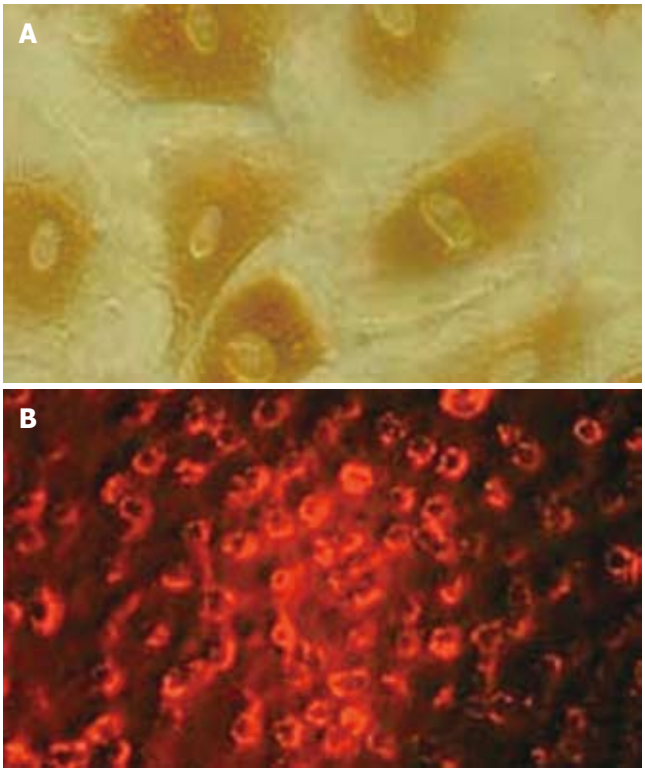


Figure 3 Immunofluorescence study of cultured human endothelial cells. **A**: Shows immunofluorescence positive-stained cells treated with Factor Ⅷ related antigen. The cells were stained brown (× 400); **B**: Shows positive-stained cells *in vitro* treated with DiI-Ac-LDL. More than 95% HUVECs are found to be red fluorescence (× 400).

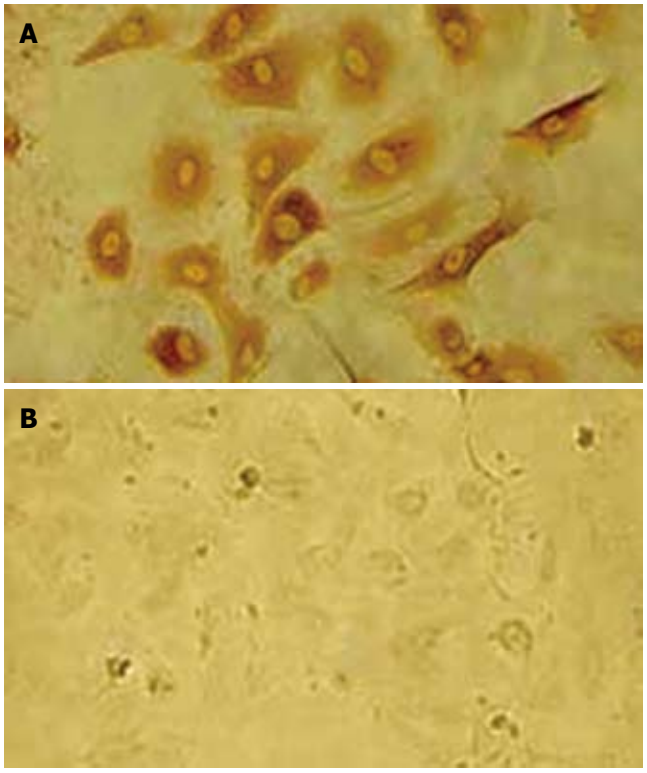


Figure 4 Immunofluorescence study (× 400) of cultured human endothelial cells. **A**: Shows positive-stained human endothelial cells with KDR receptor; **B**: Shows negative-stained human hepatoma cells without KDR receptor.

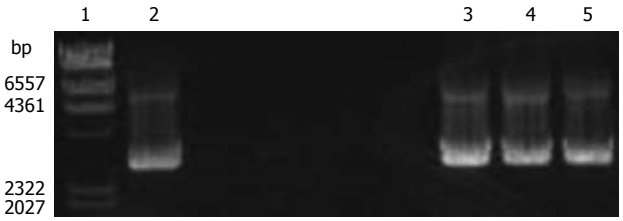


Figure 5 Identification of recombinant pEGFP-KDR-TK by restriction enzyme digestion (*Sal*Ⅰ/*Xho*Ⅰ) and electrophoresis. Lane 1: DNA markers (λDNA/*Hind*Ⅲ); Lane 2-5: pEGFP-KDR-TK was digested with *Sal*Ⅰ/ *Xho*Ⅰ.

revealed that the cells in group A had no obvious coloration (Figure 6A). Fluorescent staining was observed in few cells (1.7%) in group B (stimulated by ultrasound only, Figure 6B), whereas the HUVECs transfected by the pEGFP-KDR-TK in group C (stimulated by both SonoVue and ultrasound exposure) showed clear green fluorescence within 20.3% HUVECs indicating EGFP expression (Figure 6C and D). EGFP and TK were co-expressed in HUVECs by microbubble-enhanced ultrasound irradiation, followed by selection of permanent transfectants in the presence of G418. The G418-resistant cells were named KDR-TK/VEC.

Cell survival rate

The percentage of surviving cells was examined by trypan blue dye exclusion after ultrasound exposure. The cell survival rates were 94.0% ± 1.1% in the group A, 93.5% ± 0.9% in the group A and 90.2% ± 0.9% in group B (Figure 7).

Sensitivity of genetically modified cells to GCV

We selected GCV as a prodrug and examined the sensitivity of KDR-TK/VEC, HUVEC, and HepG2 to GCV. As shown in Figure 8, HepG2 cells were found to be resistant to GCV, and the value of IC 50, defined as the dose required for 50% cytotoxicity, was > 100 μg/mL (the highest concentration tested). HUVECs exhibited a higher sensitivity to GCV in comparison to HepG2, when the concentration tested was above 50 μg/mL, the IC 50 value for HUVECs was still more than 100 μg/mL. Conversely, KDR-TK/VEC cells were susceptible to GCV in a dose-dependent manner and exhibited approximately 75% suppression at a GCV concentration of 100 μg/mL, with the IC 50 value being approximately 10 μg/mL. KDR-TK/VEC cells also showed significantly higher sensitivity to GCV compared to non-transfected HUVECs, resulting in 2 to 5-fold higher suppression to GCV.

DISCUSSION

Acquired drug resistance is a major problem in cancer therapy. Many deaths from cancer may follow the development of resistance to chemotherapy. The emergence of resistance depends in part on the genetic instability, heterogeneity, and high mutational rate of tumor cells[1]. In contrast, endothelial cells have relative genomic stability. Therefore, antiangiogenic therapy targeting tumoral endothelial cells may avoid the problem

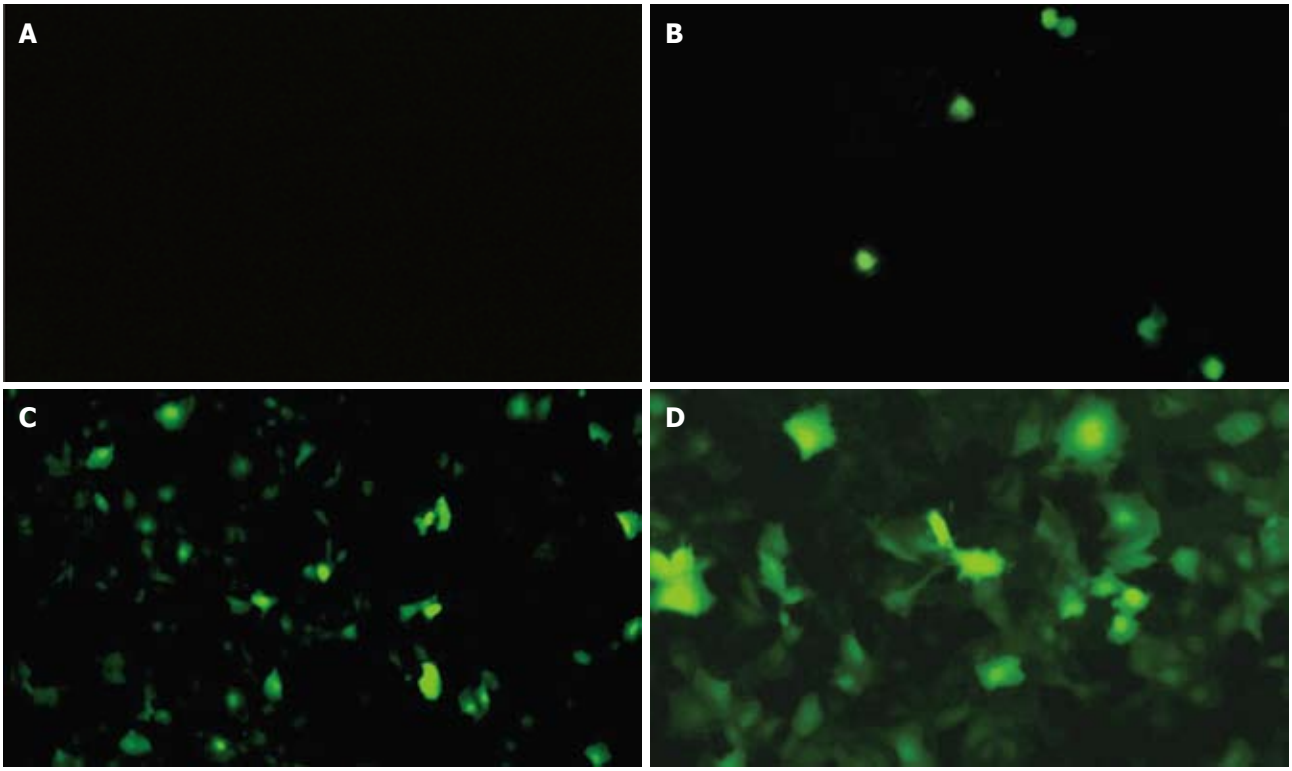


Figure 6 Fluorescent microscopy showed EGFP expression 48 h after pEGFP-KDR-TK transfection. **A**: Shows no obvious EGFP expression in cells without microbubbles and ultrasound (× 200); **B**: 1.7% cells exposed to ultrasound only shows EGFP expression (× 200); **C, D**: Significant increase in EGFP expression in HUVECs exposed to both the ultrasound and SonoVue (**C**: × 200, **D**: × 400). About 20.3% HUVECs had EGFP expression.

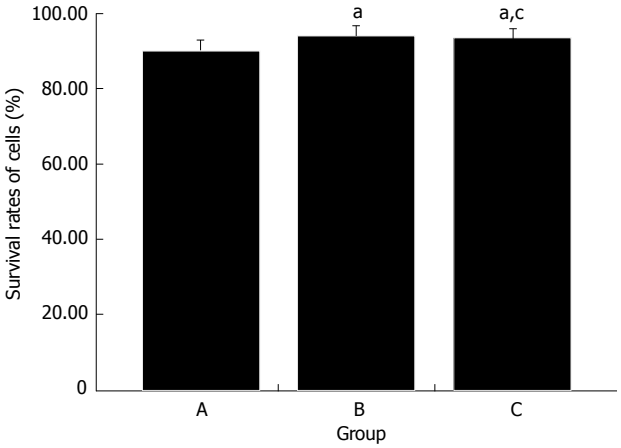


Figure 7 HUVECs survival rate examined by trypan blue dye exclusion after transfection. Values are expressed as the average percentage of viable cells, $n = 5$ from 3 independent experiments. $^{a}P < 0.05$ *vs* group A; $^{c}P > 0.05$ *vs* group B.

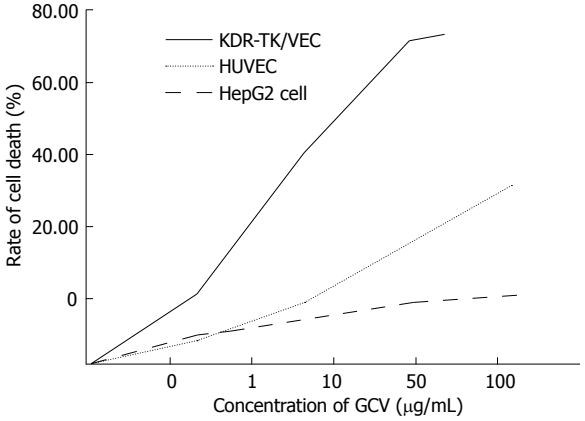


Figure 8 *In vitro* cytotoxic effect of GCV on KDR-TK transfected HUVECs (KDR-TK/VEC), non-transfected HUVECs, and HepG2 cells. KDR-TK/VECs, non-transfected HUVECs, and HepG2 cells were cultured with various concentrations of GCV for 3 d and viability was estimated by MTT assay.

of drug resistance. In addition, the growth and persistence of solid tumors and their metastases are angiogenesis dependent[11], thus angiogenesis provides a target for therapeutic approaches to solid tumor, and the inhibition of angiogenesis is very promising.

Most angiogenic inhibitors are biologically active peptides. It is difficult to express proteins persistently and stably *in vivo*, and the complexity and time requirement for protein purification make their clinical application infeasible[12]. Gene therapy is an attractive means of delivering these antiangiogenic agents to tumor site, and targeting the tumor vessels could avoid potential side effects. Although several previous studies have shown the effectiveness of TK targeting in endothelium as antiangiogenic strategy, few of them have reported with suicide gene therapy by targeting TK in the vasculature and not in the human tumor cells[13,14]. Ozalki *et al* have shown that vWF2 promoter combined with HSV-TK suicide gene can exert an endothelial-preferential killing effect on the transduced HUVECs *in vitro*[15]. Von Willebrand factor (vWF) is a blood glycoprotein which is excreted by endothelium and megakarcytes, and the elevation of vWF

is not only found in cancer, but also in other cardiovascular diseases[16]. Consequently, tumor vessels are not accessible targets for antiangiogenic gene therapy, when the vWF2 promoter and HSV-TK/prodrug are administrated systemically. Compared with vWF, KDR has a major role in tumor angiogenesis, and in adulthood, KDR appears mostly restricted to vascular endothelial/lymph endothelial cells[2]. Because of the overexpression of KDR in tumors vasculature, KDR-promoter did not yield high TK protein expression in normal vasculature, whereas in endothelial cells within tumors. Dancer and colleagues indicated that expression of TK driven by an endothelial-specific promoter can cause significant decrease in microvessel density and inhibit tumor growth[17].

The suicide gene used in this study has been widely used in gene therapy of animal tumors, and it encodes an enzyme that can effectively phosphorylate the antiviral nucleoside analogue ganciclovir (GCV) to its monophosphase form, which is further phosphorylated by cellular kinases to DNA polymerase inhibitors[18]. Apoptosis has been demonstrated as the major mechanism of cell death caused by the HSV-TK/GCV system in different cells[19,20]. The present study demonstrated that KDR promoter and the suicide gene/prodrug system showed a targeted killing effect on transfected HUVECs. The sensitivity of KDR-TK-transfected HUVECs to GCV was found to be about 2 to 5-fold higher when compared to non-transfected HUVEC and HepG2 cells.

Viral delivery systems have been developed as highly efficient carriers for gene delivery to a variety of tissues. However, viral gene carriers have some major disadvantages such as possible immune response, severe inflammation reactions, the risk for insertional mutagenesis, and interference with expression of cellular genes[21,22]. Recently, ultrasound together with microbubble has been recognized as a useful means for controlled delivery of drugs. Although the mechanism of enhanced transfection efficiency by ultrasound is not yet fully understood, cavitation is an important event that increase capillary permeability and produce transient nanopores in cell membranes. SonoVue as well as other microbubble can be rapidly destroyed by the sonication, and the threshold of cavitation decreases after addition of microbubbles. In this study, we demonstrate that widely used diagnostic ultrasound and microbubble-based ultrasound contrast agent can also be applied for the delivery of DNA. The expression of EGFP in HUVECs was just 1.7% in the group only treated with ultrasound, whereas the expression rate significantly increased to 20.3% ($P < 0.001$) when treated with ultrasound and SonoVue. In contrast, therapeutic or high-intensity focused ultrasound has been used for most of the described ultrasound-facilitated gene delivery and gene transfection experiments[23-25]. An advantage of using diagnostic ultrasound instead of therapeutic ultrasound allows visualization of the site of interest and microbubble possible by using conventional ultrasound imaging systems. In this study, more than 90% of the cells treated with ultrasound and SonoVue were viable. Though the longer exposure time used in our experiment, the cell viability in this study is also higher than that using therapeutic ultrasound[26]. Although the transient transfection rate of KDR-TK in this study is relatively low compared with other studies using viral vectors, we believe this disadvantage can be offset by lower acoustic frequency, higher ultrasound output, or longer exposure time.

Our observation also suggested that non-transfected HUVECs have more sensitivity to GCV, compared with non-transfected HepG2 cells. The mechanism was not yet understood; we speculated that the lower sensitivity to GCV of non-transfected HepG2 cells might partly involve the deficiency of TK in tumor cells[27].

In summary, our study revealed that, on the basis of the KDR-mediated invasion, TK/GCV can accomplish a selective killing effect on HUVECs *in vitro*. KDR promoter and the suicide gene/prodrug system mediated by diagnostic ultrasound combined with microbubble may present a novel and attractive approach for gene therapy to target tumor vessels.

COMMENTS

Background

Tumor angiogenesis plays a critical role in the development and progression of the solid tumor. Targeting angiogenesis represents a new strategy for antitumor therapies. Ultrasound exposure in combination with microbubble is a new non-viral delivery system.

Research frontiers

Gene therapy to target tumor vessels can cause a significant decrease in microvessel density and inhibit tumor growth. Just as therapeutic ultrasound, diagnostic ultrasound in conjunction with microbubbles can also be a useful gene delivery system.

Innovations and breakthroughs

Diagnostic ultrasound-facilitated gene delivery on targeting the vascular endothelial cells has not been used. This study established a specific promoter in order to target gene expression in human umbilical vein endothelial cells and demonstrated that diagnostic ultrasound combined with microbubble can be a useful gene delivery system.

Applications

This study showed KDR promoter and the suicide gene/prodrug system mediated by diagnostic ultrasound can significantly kill human umbilical vein endothelial cells. This may present a novel and attractive approach to target gene therapy on tumor vessel.

Terminology

Microbubble: microscopically gas bubbles encapsulated by an elastic shell, can be used in diagnostic and therapeutic applications.

Peer review

This study is well-designed and very interesting, and the manuscript is well-written.

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CLINICAL RESEARCH

Clear cell colitis: A form of microscopic colitis in children

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Abstract

AIM: To describe a new clinical and pathological subtype of microscopic colitis in children.

METHODS: A selected group of children with abdominal pain, constipation and/or diarrhoea showing discrete or no macroscopic abnormalities on endoscopy was described.

RESULTS: Multiple biopsies of colon showed large mononuclear clear cells in lamina propria of mucous membrane provided that good quality histological sections were performed and observed under a higher magnification. Otherwise, they could be misinterpreted as artefacts. Their presence in routine histology might suggest a systemic storage disease (Whipple's disease), and neuronal intestine dysplasia. Using immunohistochemical staining and electron microscopy we confirmed their origin from CD68 positive mononuclear macrophages.

CONCLUSION: The presence of large clear cells is a constant microscopic feature. Failure of transient large bowel stationary macrophages plays a role in the pathogenesis of this benign microscopic clear cell colitis, sometimes coexisting with allergy.

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Key words: Microscopic colitis; Clear cell colitis; Diarrhoea; Constipation; Abdominal pain; Children; Allergy; Endoscopy

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INTRODUCTION

Microscopic colitis constitutes a group of diseases with unknown aetiology and causes neither destruction nor rebuilding of large bowel mucosa. Microscopic colitis is rarely recognized in children[1,2] and may be misinterpreted as inflammatory bowel disease[3]. Various forms of microscopic colitis have been identified in adults[4-11], including lymphocytic, collagenous, multinucleated giant cells. No description of microscopic colitis with the presence of giant mononuclear clear cells in lamina propria in children is available. Several staining methods can be used to identify the phenotype of these cells. This is probably not a new disease but it is frequently overlooked due to routine use of low magnifications and poor quality of histological staining for paraffin-embedded materials. The presence of large amounts of these cells in our series of biopsies is characteristic in children with chronic abdominal pain, diarrhoea or constipation. In three cases, the preliminary diagnosis was Whipple's disease, neuronal dysplasia and even Gaucher disease. Definitive diagnosis of clear cell colitis (CCC) was made by immunohistochemistry methods and electron microscopy. A higher diagnostic profile is possible for identification of various forms of microscopic colitis.

Clinical, endoscopic and morphological analyses of CCC were described in series of large bowel biopsies. Characteristic giant mononuclear clear cells were found in sections by routine hematoxylin and eosin staining. Magnifications larger than 200 × must be used and high quality paraffin histology sections must be obtained to detect the cells. Otherwise, CCC may be overlooked. Using immunocytochemical and electron microscopic procedure, these clear cells were identified as activated macrophages which were CD68 positive and contained multiple vacuoles with characteristic amorphic homogenous lipid like material. The cells were found only in various parts of large intestine but not in small intestine or gastric or duodenal mucosa. Ten-year observation of patients in whom repeated biopsies were performed, allowed us to conclude that CCC is a form of microscopic colitis with chronic persistent benign course and persistent constant histological pattern. During the last 3 years, 20 patients underwent a prolonged (6-mo to a 1-year) therapy with probiotics. Clinical symptoms improved and the number of clear cells decreased in microscopic examinations. In 6 cases the cells disappeared

completely from mucous membranes. The remaining patients received a continuous long-term follow-up.

MATERIALS AND METHODS

Patients

Clinical and morphological study on CCC involved 81 children aged between 17 mo and 17 years selected out of 5000 large bowel biopsies taken from children who underwent treatment in paediatric centres between 1994 and 2005. The group included 63% boys and 18% girls. The majority of patients (68%) were inhabitants of urban areas, whilst the rest (32%) lived in the countryside. Colon dysfunction was the reason for diagnostic investigation. The majority of patients reported recurring abdominal pain, diarrhoea, constipation, and poor appetite. Their stools were of various consistency and contained mucus. Each child had at least two endoscopic examinations of the final part of the alimentary tract (23 had rectoscopy and 58 had colonoscopy).

Clinical, laboratory and endoscopic evaluations were performed carefully. Clinical diagnosis was established based on the number of stools per week, the presence of mucus, general comfort of a child, intensity of stomach aches, the presence of fever, ESR, haemoglobin concentration, state of nutrition and the presence of parenteral manifestations. Evaluating the rate for changes in endoscopic image, we took into consideration vascular drawing, granulation of mucous membrane.

Bacteriology and virology

The presence of intestinal pathogens (Salmonella, Shigella, Campylobacter, Escherichia coli, Yersinia, Clostridium difficile) and Giardia infection was verified. Diagnostic research for the presence of enteroviruses was performed in the Center of Virology of the National Institute of Hygiene on the Hep-2 and RD cell lines supplied by the WHO. The presence of retrovirus was checked in order to obtain a full virological evaluation. Tests of the latex reaction with Slidex Rota-kit 2 set and ELISA serological examination (using the DAKO rabbit anti-rotavirus (Human) reactants recommended by the WHO) were performed.

Morphological examination

During colonoscopy 3 specimens were collected from each of the 58 patients. Colonoscopy was performed 2 times and a total number of 248 biopsies were examined. A comparison of long-term changes occurring in the course of CCC was possible. Rectoscopy was performed 3 times in 23 cases. A total number of 317 biopsies were collected. Paraffin-embedded specimens were cut into serial sections which were stained with hematoxyllin and eosin. In 81 cases, 162 immunological staining procedures were performed with 16 monoclonal antibodies (to CD3, CD4, CD22, CD34, CD31, CD68, PCNA, Ki67, chromogranin, synaptophysin, neurofilament, actin, p53, GFAP, IL-beta, TNF alpha) and 4 histological staining procedures were performed for mucicarmin, PAS, PAS diastase. A total of 2916 staining procedures were reviewed (Table 1). The phenotypes of clear cells were identified.

Table 1 Expression of immunocytochemical markers in the CCC of the colon submucosa including some histological staining

Marker	Clear cells
CD3 (T cell)	Negative
CD4 (T cell)	Negative
CD22 (B cell)	Negative
CD34 (precursor cell)	Negative
CD31 (Endothelial)	Negative
CD68 (Macrophages)	+++ Strongly positive
PCNA-proliferation antigen	Negative[2]
Ki67 mitotic activity	Negative[1]
Chromogranin	Negative
Synaptophysin	Negative
Naurofilament	Negative
Actin	Negative
P53 mutations	Negative
GFAP glial marker	Negative
TNF alpha	Negative
IL2	Negative
Mucicarmin	Negative
PAS	Strong positive
PAS diastase	Digested
Osmium tetraoxide	Positive

[1]1-3 per crypt; [2]Positive in the cryptic epithelium.

In 10 cases, examination under an electron microscope (Jeol 100CX) was also performed. Preparations of intestinal mucosal biopsies were fixed in 4% glutaraldehyde in cacodylate buffer and then in OsO_4, followed by a typical Epon procedure. Ultra-thin sections were counterstained with lead citrate and uranyl acetate.

RESULTS

Clinical observation

The incidence of CCC as a form of microscopic colitis (MC) was 2% in children with abdominal pain, diarrhoea and constipation. ESR < 100 mm and MHC/hemoglobin concentration > 8.5 g/dL were found in all children. During the 10 years of observation, no clinical or endoscopic changes in IBD were noticed. Before analysis, children did not take any medicines or drugs that could caused pathological changes in mucous membrane of the large intestine. No treatment was administered. In some cases, a pre-treatment with salazopyrine for a few months did not result in regression of endoscopic or histological changes. Clinical symptoms were improved when probiotics was administered in 20 patients. Clear cells disappeared on mucosal membranes in the second biopsy from 6 patients. The remaining patients remained on treatment with Saccharomyces boulardi.

The dominant symptoms in both girls and boys included recurrent abdominal pain, stiff and (or) loose stools, even diarrhoeal stools with mucus additive. Some patients were admitted to gastroenterology hospital departments mainly because of suspected IBD. Some showed atopic skin inflammation and (or) recurring upper respiratory system inflammation. Five children with bronchial asthma were treated. Laboratory peripheral blood and urine tests and microbiologically parasitological stool tests did not reveal any pathological changes.

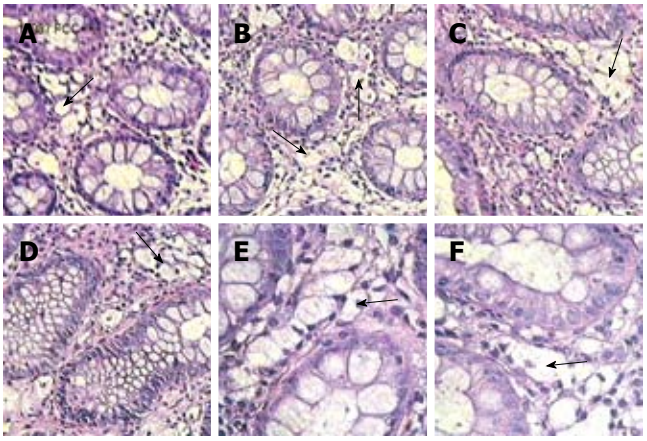


Figure 1 Microscopic features of CCC (**A-F**) (HE, × 320).

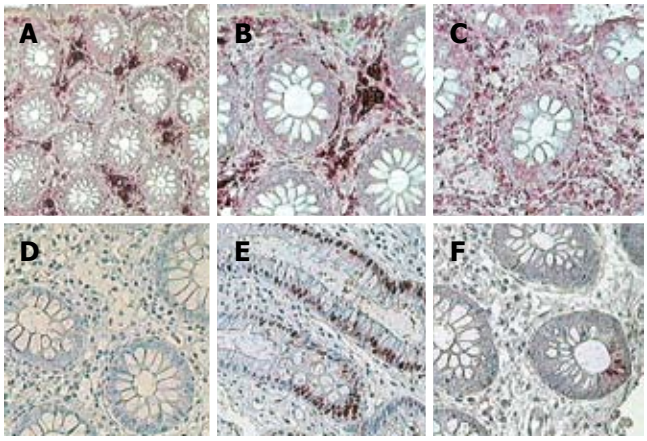


Figure 2 Immunocytochemistry of CCC using monoclonal antibodies and APPAP method showing positive expression of mononuclear phagocytic cell line (**A** and **B**), negative T3 and T4 lymphocytes (**C**), negative neurofilament (**D**), Normal PCNA expression in cryptal epithelium (**E**), and normal mitotic activity of positive CD67 (**F**) (× 320).

Abdominal pain occurred in all cases. The pain was mild or moderate. Less than 18 stools per week were counted in 7 children, 18-35 stools in 59 cases and 36-60 stools per week in the remaining 15 cases, constipation in 19 cases. A subjective evaluation of general feeling of children was good. Parenteral symptoms were noted in 19 out of 81 children, mainly upper respiratory tract infection. Forty-three children had ESR less than 40 mm, 20 had 50-100 mm ESR after 1 h. Haemoglobin concentration was above 10 g/dL in all cases. The state of nutrition (Cole and Stanfield) was over 85% in 27 cases, 80%-85% in 46 cases and under 80% in 8 cases. Bacteriological and virological examination of stools gave negative results.

Endoscopic examination

Endoscopic examinations were done with a stiff rectoscope. Eighty-two percent of the patients had also a supplementary colonoscopy. Macroscopically, all patients appeared to have normal mucosa or granulated mucosa, with sharply outlined vascularization and a thin layer of mucus in the rectum and sigmoid colon. In children with CCC undergoing colonoscopy, characteristic fine granulation and increased sharpness of vascular outline in the descending and transverse colon were found and changes in the ascending colon were also observed in 6 children. Susceptibility to bleeding was increased in 9 children. Three to six biopsies were taken during each endoscopic examination of the large intestine, observation was repeated 3 times during the 5-10 years. One specimen was collected at each rectoscopy during the 5-10 years of observation. In the consecutive rectoscopic and endoscopic examinations, the mucosal pattern of CCC was not changed. Persisting granulation and increased sharpness of the vascular pattern were revealed. Endoscopic examination of the upper part of alimentary tract gave normal results.

Histology

Microscopic images of routinely HE-stained sections were analysed. There were large clear cells in the lamina propria of mucosa, appearing either separately or in groups (Figure 1). The cells occupied the entire lamina propria from the covering epithelium to the basis of cryptal glands and the level of muscularis mucosa. Distribution of the clear cells allowed identification of 3 degrees of clear cell density: grade 1-single cells at various levels of lamina propria, grade 2-the presence of aggregates of 3-10 cells, grade 3-clear cells uniformly distributed in lamina propria. The presence of clear cells in patients was permanently observed during the 5-10 years. Clear cells showed a positive reaction with neutral mucopolisacharides in the PAS method with periodic acid and the Shiff's reagent (PAS- positive reaction), and negative reaction after digestion with diastase. Staining with mucicarmine was negative. No intraepithelial lymphocytosis was noted and the base membrane was normal. About 243 serial sections stained with HE were reviewed microscopically.

Immunocytochemistry

Clear cells presented a strong positive expression in the reaction with the CD68 antibody (Figure 2A-F, Table 1). Sixteen immunocytochemical reactions were performed 3 times in 81 patients during the period of observation in order to identify the phenotype of large clear cells.

Electron microscopy

In contrast to primary metabolic diseases, electron microscopic examination of clear cells revealed the presence of single nuclei with nucleoli and multiple secondary phagosomes with accumulation of homogenous osmophilic materials (Figure 3). Other structures of the large intestine mucosa were normal. The material for electron microscopic analysis was taken from 10 patients with CCC confirmed previously by histological examination.

DISCUSSION

Microscopic colitis is the term suggested by some authors for abdominal pain, alternating chronic diarrhoea and constipation and endoscopic image close to normal[4-8]. A characteristic microscopic picture of adults is in the form of collagenous and lymphocytic colitis[9-11]. The cause for microscopic colitis is unknown. Several concepts are

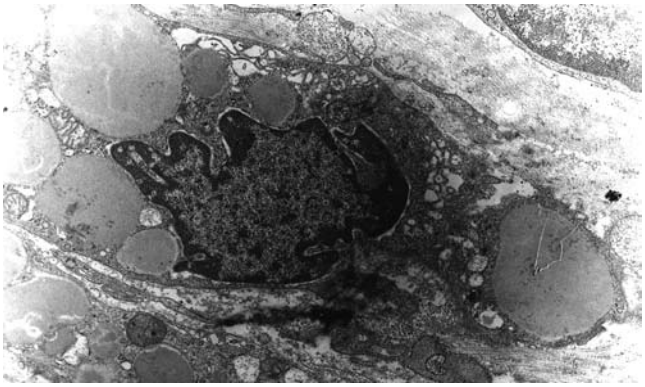


Figure 3 Electron microscopy of clear cells. Giant clear macrophages contain multiple vesicles varying in size filled with homogenous osmiophylic materials (secondary phagosomes) without any structural components in their cytoplasm (OsO_4 staining, × 24 000).

published, including abuse of medications[12], increased concentration of antigens and cytokine expression[13], auto-antibodies in blood[14], prostaglandin level[15]. MC may overlap with indeterminate inflammatory bowel disease[16], but in contrast to CCC, TNF alpha expression may be positive in silent inflammatory bowel disease[17].

CCC in children as a form of microscopic colitis is characterized by the presence of various large mononuclear clear cells in lamina propria. The cells are easily recognized when high magnification microscopy is used and section staining of microscopic slides is good. CCC does not manifest as destruction or reconstruction of glands. In the present study, basic membrane was not damaged, no lymphocytic or calagenous colitis or other forms of microscopic colitis described elsewhere were observed. Clear cells are mononuclear in contrast to other microscopic colitis, where there are giant multinucleated cells[18]. Clear cells described here are different from other muciphages[19]. On the other hand, Hamrock[20] has reported clear cells in patients with AIDS. The cells look like Whipple cells but they can be overloaded with inclusion material (Mycobacterium). Some authors suggested that rectal biopsy should be performed in cases of irritable bowel disease since they may be classified as MC[21].

Differential diagnosis should also consider neuronal dysplasia[22] of intestine, in which large, bright ganglion cells can be very similar to the giant clear cells in CCC. The reaction with neurofilaments, synaptophisine or (and) chromogranine is helpful. Some authors suggest that Salmonella typhimurium type can mobilize phagocytosis in macrophages of the large intestine but we have not found bacterial or viral particles in the phagosomes of clear cells. Different macrophages described in Erdheim-Chester disease exhibit neoplastic characteristics[23]. Possible persistent changes (e.g. after the rotaviral diarrhoea) and the presence of enteroviruses in clear foamy cells can be ruled out on the ground of electron microscopy and negative results of blood and stool test.

Xanthelasma of rectum containing sialomucin, is a different disease rather than CCC. However, patients with similar symptoms suffer from diarrhoea, constipation or haemorrhages. The presence of these symptoms is a result of mechanical injuries of mucosa in the course of chronic constipation. In contrast to muciphages, clear cells were negative for mucicarmine staining in our study.

The mononuclear phagocytic system consists of macrophages widely distributed in the body as circulating or stationary cells. Macrophages of large bowel mucosa belong to stationary cells and have various functions. In some special unknown conditions, they can transform into large clear cells and exhibit transient malfunction with accumulation of lipid material. It should be emphasized that one third of our patients had allergic extra intestinal manifestations. Finally, application of probiotics (Saccharomyces bulardi) has a beneficial effect in children with CCC. Confirmation of this finding requires further long-term observation.

COMMENTS

Background

Clear cell colitis (CCC) is a description of a new form of microscopic colitis in children. This is a benign disease recognized in some children showing symptoms of recurrent alternate diarrhoea and constipation. Patents are directed by physicians to gastroenterology units of district hospitals. Endoscopic examination shows normal or minor changes which do not constitute a qualification for further therapy. Pathologists using usually low magnifications can overlook the presence of large clear cells in lamina propria, and describe mucosa as normal. The lesions are seldom found in academic gastroenterological centers, where selected patients with more serious symptoms are ordered.

Research frontiers

Detection of giant clear cells is very important if no other lesions in large intestine musosa or other organs are found. Otherwise, false diagnosis of primary metabolic disease or neuronal displasia can be made. Before application of immunocytochemical tests, electron microscopy is useful in differential diagnosis, even microscopic examinations of conjunctiva and liver are recommended.

Innovations and breakthroughs

The reason why giant clear cells are aggregated is not clear. The presence of so called muciphages, described previously as a phenomenon in adults can be excluded on the basis of negative mucicarmine staining and different electron microscopic pictures.

Applications

As the children included in the study showed symptoms of allergy, and even asthma, selective activation of lipid and protein storing macrophages is considered to be associated with mucosa auto-immunological response, probably under influence of antigenemia occurring in children suffering from constipation. It should be emphasized that the presence of giant macrophages is chronic and the clinical course is stable. In the present paper, no therapy was needed at the end of a long term clinical and morphological observation period. During the last two years, some improvement was achieved after application of probiotics. Usage of probiotics (among others of Lactobacilus casei) in all children is the aim in future studies.

Terminology

Clear cell colitis is a form of microscopic colitis in children with accumulation of giant clear cells.

Peer review

This manuscript appears to be reasonably well written. The authors described a new form of CCC in children and presented evidence to support their contention.

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RAPID COMMUNICATION

Elevated nitric oxide and 3',5' cyclic guanosine monophosphate levels in patients with alcoholic cirrhosis

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Abstract

AIM: To evaluate whether serum levels of nitric oxide ($NO^{\bullet}$) and plasma levels of cyclic guanosine monophosphate (cGMP) and total glutathione (GSH) are altered in patients with alcoholic cirrhosis and to examine their correlation with the severity of liver disease.

METHODS: Twenty-six patients with alcoholic liver cirrhosis were studied. Serum levels of $NO^{\bullet}$ and plasma levels of cGMP and GSH were measured in 7 patients with compensated alcoholic cirrhosis (Child-Pugh A) and 19 patients with advanced cirrhosis (Child-Pugh B and C). The model for end-stage liver disease (MELD) score was evaluated. Sixteen healthy volunteers served as controls. Liver enzymes and creatinine levels were also tested.

RESULTS: $NO^{\bullet}$ and cGMP levels were higher in patients with Child-Pugh B and C cirrhosis than in Child-Pugh A cirrhosis or controls ($NO^{\bullet}$: 21.70 ± 8.07 *vs* 11.70 ± 2.74; 21.70 ± 8.07 *vs* 7.26 ± 2.47 μmol/L, respectively; $P < 0.001$) and (cGMP: 20.12 ± 6.62 *vs* 10.14 ± 2.78; 20.12 ± 6.62 *vs* 4.95 ± 1.21 pmol/L, respectively; $P < 0.001$). Total glutathione levels were lower in patients with Child-Pugh B and C cirrhosis than in patients with Child-Pugh A cirrhosis or controls (16.04 ± 6.06 *vs* 23.01 ± 4.38 or 16.04 ± 6.06 *vs* 66.57 ± 26.23 μmol/L, respectively; $P < 0.001$). There was a significant correlation between $NO^{\bullet}$ and cGMP levels in all patients with alcoholic cirrhosis. A significant negative correlation between reduced glutathione/glutathione disulfide and the MELD score was found in all cirrhotic patients.

CONCLUSION: Our results suggest a role for oxidative stress in alcoholic liver cirrhosis, which is more significant in decompensated patients with higher levels of $NO^{\bullet}$ and cGMP and lower GSH levels than in compensated and control patients. Altered mediator levels in decompensated patients may influence the hemodynamic changes in and progression of liver disease.

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Key words: Oxidative stress; Nitric oxide; Alcoholic cirrhosis; Fibrosis; Model for end-stage liver disease

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INTRODUCTION

Increased production of free radicals has been implicated in several conditions, such as liver damage and fibrosis[1]. Free radicals can damage cellular macromolecules and, therefore, may participate in hepatocellular injury when produced in excess. Endotoxins or cytokines induce macrophages, neutrophils and vascular smooth muscle cells to express a calcium-independent isoform of nitric oxide synthase (NOS). Nitric oxide ($NO^{\bullet}$) is a messenger molecule that functions as a vasodilator and accounts for the biological activity of endothelium-derived relaxing factor (EDRF)[2-4]. The three isoforms of nitric oxide synthase (NOS), the enzyme responsible for the generation of $NO^{\bullet}$, are all found in the liver[4].

Production of $NO^{\bullet}$ activates the soluble guanylate cyclase (sGC) the enzyme with a prosthetic heme group by binding to the ferrous heme iron (Fe^{2+}), resulting in the formation of cyclic guanosine 3'-5' monophosphate (cGMP) from guanosine 5' triphosphate (GTP). This process occurs in both endothelial and vascular smooth muscle cells[5,6]. The increase in concentrations of the second messenger cGMP is stimulated by activation of dependent protein-kinase G (pKG) and confers signalling capacity to the free radical *via* $NO^{\bullet}$-sGC-cGMP-PKG pathway, which in turns leads to the relaxation or dilation of vascular smooth muscle cells (VSMCs), and, subsequently, to inflammation and tissue damage mediated by reactive oxygen species (ROS; for example, $O_2^{\bullet}$ and $OH^{\bullet}$).

$NO^{\bullet}$ promotes tissue toxicity when it reacts with the superoxide anion radical ($O_2^{\bullet}$), which leads to formation of peroxinitrite anion ($ONOO^-$) and hydroxyl radical ($OH^{\bullet}$)[6-9] ($NO^{\bullet} + O_2^{\bullet} \rightarrow ONOO^- + OH^{\bullet}$). Both of these products $OH^{\bullet}$ and $ONOO^-$ are aggressive free radicals and powerful oxidants that can cause membrane lipid peroxidation[5-7,10]. Antioxidants are substances that help to reduce the severity of oxidant stress either by contributing to the formation of a less active radical or by quenching the reaction of $NO^{\bullet}$ which produce peroxinitrite anion and hydroxyl radical. Glutathione (GSH) and its related enzymes are protective effects against oxidative damage[11]. One consequence of GSH depletion, as seen during redox stress, would be enhanced peroxinitrite formation. Glutathione may also be involved in the detoxification of peroxinitrite. Both $O_2^{\bullet}$ and $OH^{\bullet}$ increase production of ROS and GSH depletion, which leads to the abnormal breakdown of fat molecules (that is, lipid peroxidation). This complex process results in the formation of toxic compounds and may contribute to the pathogenesis of alcoholic liver injury.

The aim of this study was to investigate whether serum levels of $NO^{\bullet}$ and 3',5'cyclic guanosine monophosphate (cGMP), and plasma levels of thiol-reduced glutathione (GSHr) and glutathione disulfide (GSSG), are altered in patients with alcoholic liver cirrhosis, and to assess their possible correlation with the severity of liver disease, measured by the Child-Pugh and MELD scores.

MATERIALS AND METHODS

Patients

This study was performed in 26 patients with alcoholic cirrhosis (19 men and 7 women, mean age 56.00 ± 12 years) admitted to the Liver Unit and Division of Gastroenterology, Santa Maria Hospital for evaluation. All patients had a history of alcoholic consumption greater than 80 g/d for at least 5 years. The diagnosis of liver cirrhosis was based on clinical, biochemical and ultrasonographic findings or histological criteria. The patients were classified as having compensated (n = 7) or decompensated liver cirrhosis (n = 19). According to the Child-Pugh scores, all compensated patients belonged to class A. The decompensated group consisted of 8 patients in class C and 11 patients in class B with a Child-Pugh score above 8. At the time of the study no Child A patients showed clinical features of decompensated liver cirrhosis (ascites, edema, encephalopathy or recent portal hypertension associated gastrointestinal bleeding). All patients graded Child-Pugh B-C had moderate to severe ascites without evidence of infection. The main clinical and laboratory findings are shown in Table 1. There was no evidence of hepatocellular carcinoma, cardiac, renal or respiratory failure in the patients studied, and none of them were administered vasoactive drugs or antioxidants during the study.

The control group consisted of 16 healthy subjects (10 men and 6 women, mean age 38 ± 18 years) with normal laboratory findings. Informed consent to participate in the study was obtained from each patient.

Methods

Peripheral venous blood from fasted healthy volunteers and fasted cirrhotic patients was collected in separate tubes, one containing the anticoagulant ethylenediamine tetraacetic disodium (Na_2EDTA) and the other without serum anticoagulant. The blood was allowed to clot for 30 min at 25℃, centrifuged at 2000 × g for 15 min at room temperature, and the serum was then separated and aliquoted into tubes for storage. To obtain plasma samples, the blood was centrifuged immediately at 2000 × g for 10 min at 4℃, and then aliquoted into tubes. The tubes were then stored frozen at -80℃ until they were used to study different biomarkers.

Analytical procedures

Determination of NOx levels: The level of serum $NO^{\bullet}$ was obtained indirectly by measuring the levels of its metabolites nitrate (NO_3^-) and nitrite (NO_2^-). The levels of the final products of $NO^{\bullet}$ oxidation (NO_3^- and NO_2^-) were assessed using the Griess reaction. NO_3^- was enzymatically converted into NO_2^- in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavine adenine dinucleotide (FAD) and nitrate reductase. Nitrite reacts with Griess reagent (1 g/L sulphanilamide 0.1 g/L N-(1-naphtyl)-ethylenediamine and 5 g/L phosphoric acid) to give a red-violet diazo dye. The determination of these two elements (NO_3^- and NO_2^-) was measured in triplicate in a microplate reader on the basis of absorbance in the visible range at 540 nm. The concentrations of NO_2^- were determined from a calibration curve obtained using standard NO_2^- solutions. The mean values of the measurements were taken as the final result. The limit of detection of the method is 0.28 μmol/L for nitrate and 0.32 μmol/L for nitrite.

Determination of cGMP: The plasma concentration of cGMP was determined in duplicate by radioimmunoassay (RIA) using a commercial kit according to the manufacturer's protocol (IBL Hamburg, Germany).

Determination of GSHt, GSSG and GSHr: For 1 mL of plasma was used (50 μL of 5% sulphosalicylic acid) to prevent spontaneous oxidation of GSH. After centrifugation at 2000 × g for 10 min, 100 μL of the supernatant was removed for assay of GSH.

The sulfhydryl group of GSH reacts with Ellman's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) to produce yellow 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GS-TNB (between GSH and TNB) is subsequently reduced by glutathione reductase and the reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH), releasing a second TNB molecule and recycling the GSH. The rate of TNB production is directly proportional to this recycling reaction, which, in turn, is directly proportional to the concentration of GSH in the sample. GSH is easily oxidized to GSSG by glutathione reductase (GR), according to the following formula:

$GSSG + NAD(P)H \rightarrow GSH + NADP^+$.

Table 1 Laboratory findings in the patients with alcoholic cirrhosis

	Child-Pugh A (n = 7)	Child-Pugh B (n = 11)	Child-Pugh C (n = 8)	Reference values
LDH U/L	403.71 ± 115.32	453.80 ± 126.10	369.25 ± 151.89	240-480
AST U/L	59.57 ± 51.56	109.20 ± 97	88.88 ± 97.81	0-31
ALT U/L	40.86 ± 27.39	58.50 ± 47.58	43.63 ± 47.43	0-31
γGT U/L	271 ± 190.32	280.12 ± 237.87	196.75 ± 160.35	5-36
AP U/L	134.57 ± 77.62	99 ± 24.61	103.88 ± 44.25	35-104
Leukocytes/μL	5784.29 ± 1677.52	7429 ± 3987.49	5731.25 ± 2187.02	4000-11000
Neutrophils/μL	3289.14 ± 1987.81	4424 ± 3042.67	3555 ± 1781.55	1900-7500
Creatinine mg/dL	1.17 ± 0.47	1.41 ± 0.54	1.91 ± 0.61	0.7-1.1
Bilirubin mg/dL	1.06 ± 0.10	1.56 ± 0.88	2.45 ± 1.37	0.1-1.1
INR	1.02 ± 0.10	1.54 ± 0.71	2.59 ± 0.99	0.8-1.1
MELD	8.29 ± 2.75	16.10 ± 6.77	23.38 ± 7.09	6-8

LDH: Lactate dehydrogenase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyltransferase; AP: Alkaline phosphatase; INR: International normalised ratio of prothrombin time. The results are expressed as means ± SD and range.

Table 2 Levels of $NO^{\bullet}$, cGMP, GSHt, GSSG, GSHr, MELD and GSHr/GSSG among the controls and patients with alcoholic liver cirrhosis (Child-Pugh A, B and C) studied

Biomarkers	Controls (n = 16)	Child-Pugh A (n = 7)	Child-Pugh B and C (n = 19)	P values
NO_3^- μmol/L	4.16 ± 1.36; (4.21)	6.73 ± 1.32; (6.89)	12.23 ± 4.29; (11.79)	$^b P < 0.001$; $^a P < 0.05$
NO_2^- μmol/L	3.10 ± 1.27; (2.90)	4.97 ± 1.5; (5.76)	9.46 ± 3.93; (7.80)	$^b P < 0.001$; $^a P < 0.05$
$NO^{\bullet}$ (NO_3^- + NO_2^-) μmol/L	7.26 ± 2.47; (7.31)	11.70 ± 2.74; (12.72)	21.70 ± 8.07; (18.22)	$^b P < 0.001$; $^a P < 0.05$
cGMP pmol/L	4.95 ± 1.21; (5.39)	10.14 ± 2.78; (9.0)	20.12 ± 6.62; (21.00)	$^b P < 0.001$; $^a P < 0.05$
GSHt μmol/L	66.57 ± 26.23; (59.88)	23.01 ± 4.38; (22.82)	16.04 ± 6.06; (14.30)	$^b P < 0.001$; $^a P < 0.05$
GSSG μmol/L	18.57 ± 6.39; (18.57)	9.43 ± 2.3; (9.65)	9.47 ± 4.17; (8.13)	$^b P < 0.001$; $^a P < 0.05$
GSHr μmol/L	47.78 ± 23.63; (45.19)	13.58 ± 3.7; (13.88)	6.54 ± 3.31; (6.02)	$^b P < 0.001$; $^a P < 0.05$
GSHr/GSSG	2.68 ± 1.16; (2.95)	1.52 ± 0.63; (1.50)	0.81 ± 0.42; (0.58)	$^b P < 0.001$; $^a P < 0.05$
MELD	6-8	8.29 ± 2.75; (8.00)	20.17 ± 8.45; (20.00).	$^b P = 0.001$; $^a P < 0.05$

Data were analysed by one-way analysis of variance (ANOVA) $^bP < 0.001$ between groups of each biomarker and Student-Newman-Keuls post-hoc test was used for comparison of difference of means between each parameter $^aP < 0.05$. $NO^{\bullet}$: Nitric oxide; cGMP: Cyclic guanosine monophosphate; GSHt: Total glutathione; GSSG: Glutathione disulfide; GSHr: Thiol-reduced glutathione; MELD: Model for end-stage liver disease; GSHr/GSSG: Thiol-reduced glutathione to glutathione disulfide ratio.

The concentrations of GSHr and GSSG in plasma were determined from a calibration curve using standard GSHr and GSSG solutions. Both GSHr and GSSG were measured in duplicate in a microplate reader on the basis of absorbance in the visible range at 405 nm.

Total GSHt is equivalent to the sum of GSSG and GSHr.

MELD score: The model for end-stage liver disease (MELD) score was calculated from the following equation:

$9.57 \times \log_e$ (creatinine mg/dL) + $3.78 \times \log_e$ (bilirubine mg/dL) + $11.2 \times \log_e$ (INR) + 6.43.

The maximal creatinine level considered in the MELD score is 4.0 mg/dL[12].

Statistical analysis

Statistical analyses were performed using the SPSS 12.0 statistical package (SPSS Inc., Chicago). All results are expressed as means ± SD, median values and ranges. Data were analysed by one-way analysis of variance in addition to the Student-Newman-Keuls post-hoc test. Coefficient correlations were evaluated using the linear regression analysis or Pearson correlation. Statistical significance was established at $P < 0.05$.

RESULTS

The baseline laboratory characteristics of the 26 patients with alcoholic liver cirrhosis (classified as Child-Pugh A, B and C) are shown in Table 1. The concentrations of serum $NO^{\bullet}$ and the different plasma biomarkers in the cirrhotic patients and controls are shown in Table 2. There were statistically significant differences between the 4 groups. The minimum $NO^{\bullet}$ level obtained in the control group was 3.19 μmol/L.

Patients with decompensated liver cirrhosis (Child-Pugh B and C) exhibited significantly higher serum concentrations of $NO^{\bullet}$ than both patients with compensated liver cirrhosis (Child-Pugh A) and controls (Figure 1). There was a significant difference in serum $NO^{\bullet}$ concentrations between control subjects and compensated liver cirrhotic patients. The concentrations of plasma cGMP in patients with decompensated cirrhosis were higher than in those in compensated cirrhosis, and this difference was statistically significant ($P < 0.001$). The concentrations of plasma cGMP in patients with compensated liver cirrhosis were higher when compared with those with the controls and this difference was statistically significant ($P < 0.001$) (Figure 2). Significant correlations between $NO^{\bullet}$ and cGMP levels ($r = 0.866$, $P < 0.001$), between $NO^{\bullet}$ levels and MELD scores ($r = 0.627$,

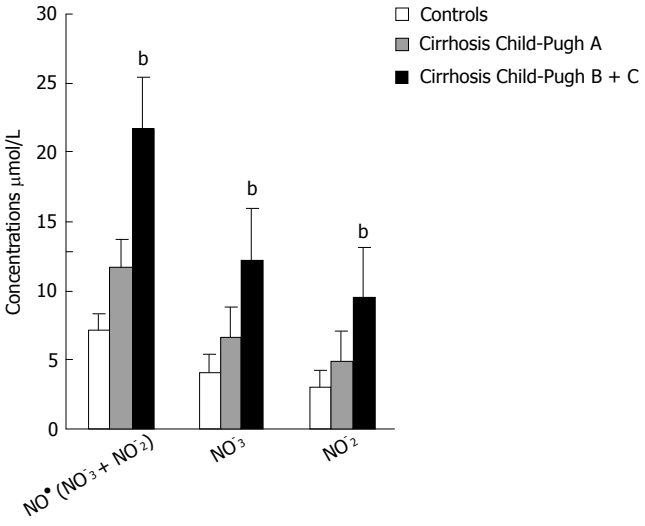


Figure 1 Serum concentrations of nitric oxide NO• (NO_3^- + NO_2^-), nitrates (NO_3^-) and nitrites (NO_2^-), in patients with alcoholic liver cirrhosis classed as Child-Pugh A, B or C, and controls. Data are expressed as means ± SD of triplicate determinations. $^bP < 0.01$ between controls and patients with Child-Pugh A, B or C cirrhosis.

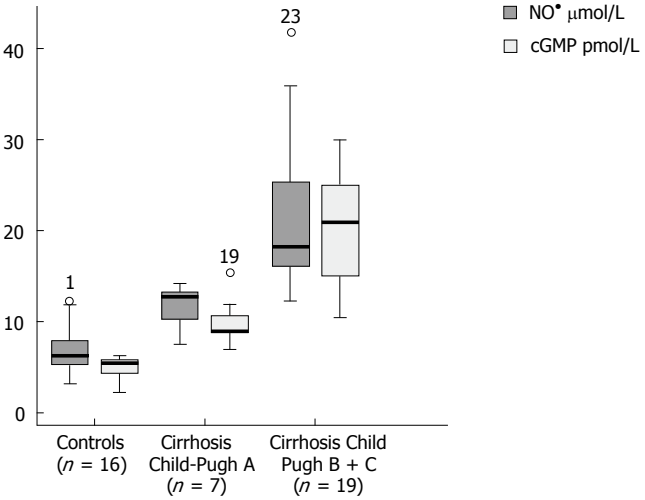


Figure 2 Boxes show the interquartile ranges. Vertical lines show 80% of the values (10^{th}-90^{th} percentiles). The bold horizontal line represents the median. $^bP < 0.01$ between controls and cirrhosis Child-Pugh A, and between controls and cirrhosis Child-Pugh B and C.

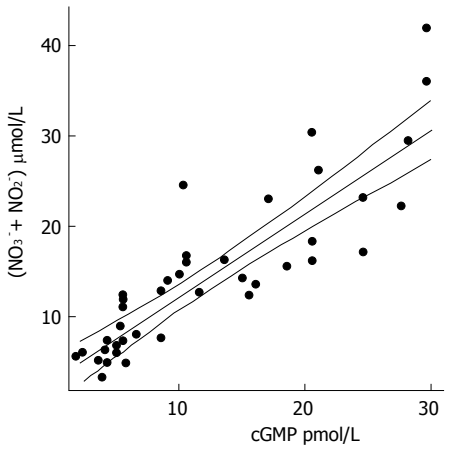


Figure 3 A significant positive correlation between the levels of NO• and cGMP was observed among the 26 patients with alcoholic liver cirrhosis, classed as Child-Pugh A, B and C. Linear regression with 95.00% mean prediction interval, $^bP < 0.01$; NO_3^- + NO_2^- μmol/L = 2.81 + 0.93; *R*-Square = 0.75.

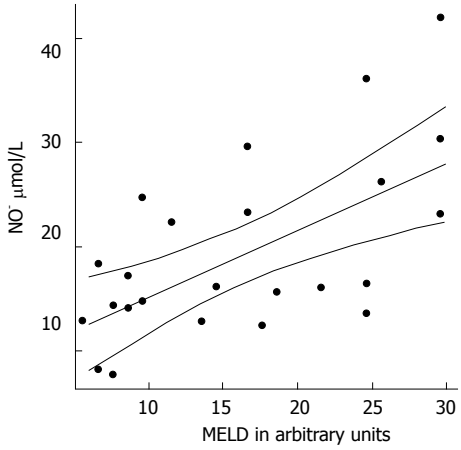


Figure 4 Correlation between NO• levels and MELD scores. Linear regression with 95.00% mean prediction interval, NO• μmol/L = 8.76 + 0.64; $^bP < 0.01$; *R*-Square = 0.39.

$P < 0.01$) and between cGMP levels and MELD scores ($r = 0.452$, $P < 0.05$) were observed among the 26 alcoholic liver cirrhotic patients (Figures 3-5).

Decreases in the plasma levels of GSHt, GSSG and GSHr were observed in all alcoholic liver cirrhotic patients, when compared with controls (Table 2). This decrease in GSHr levels was most prominent in decompensated liver cirrhotic patients. Figure 6 shows the plasma levels of the biomarkers of oxidative stress (GSHr, GSSG and GSHr/GSSG) in controls and in plasma of cirrhotic patients Child-Pugh classes A, B and C. There was a markedly reduced ratio GSHr/GSSG in patients with Child-Pugh B and C cirrhosis compared with patients with Child-Pugh A cirrhosis or controls ($P < 0.001$). The GSHr levels were higher in the controls than in the cirrhotic patients stratified according to Child-Pugh score.

The GSSG levels were higher in patients with Child-Pugh B and C cirrhosis than in those with Child-Pugh A cirrhosis or controls. The ratio between GSHr/GSSG was lower in patients with Child-Pugh B and C cirrhosis than in those with Child-Pugh A.

No correlation between NO• and cGMP levels and creatinine levels was found in patients with decompensated cirrhosis.

The MELD scores were determined in the 26 patients with alcoholic cirrhosis (Table 2). These was higher in the decompensated patients than in the compensated cirrhotic patients ($P < 0.001$). The GSHr/GSSG ratios were lower in cirrhotic patients belonging to Child-Pugh class B and C compared with those in Child-Pugh class A. Significant negative correlations between the MELD score and the GSHr/GSSG ratio ($r = -0.693$, $P < 0.001$) and between NO• level and the GSHr/GSSG ratio ($r = -0.558$, $P < 0.01$) were found in all of the alcoholic cirrhotic patients, as shown in Figures 7 and 8.

DISCUSSION

There is firm evidence of enhanced oxidative stress

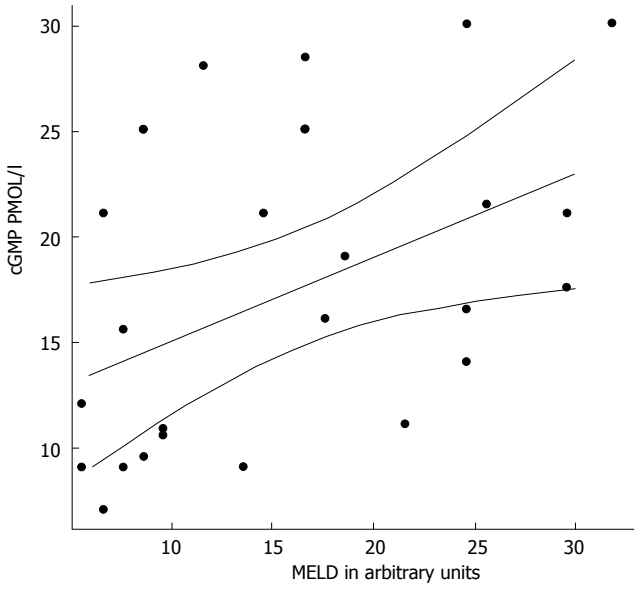


Figure 5 Correlation between cGMP levels and MELD scores. Linear regression with 95.00% mean prediction interval, $^{b}P < 0.01$; cGMP pmol/L = 11.05 + 0.40; *R*-Square = 0.20.

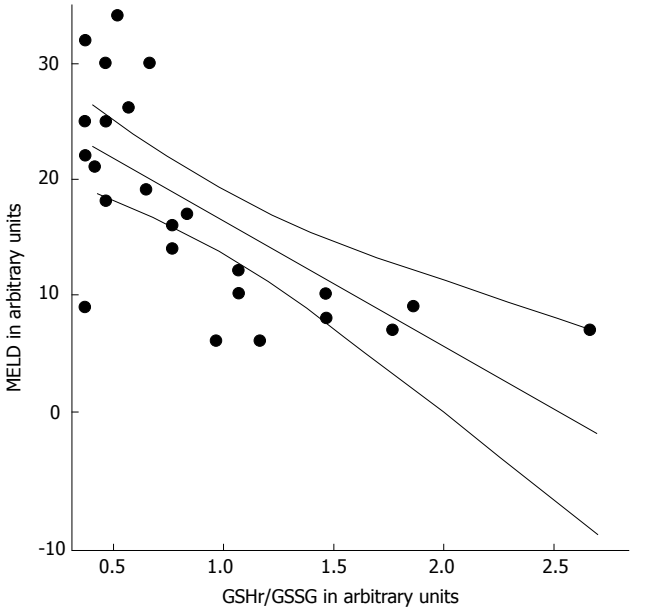


Figure 7 The MELD score increases progressively from Child-Pugh class A to C. A significant negative correlation between MELD scores and GSHr/GSSG ratios was observed. Linear regression with 95.00% mean prediction interval, $^{b}P < 0.01$; MELD in arbitrary units = 27.08 + -10.74; *R*-Square = 0.48.

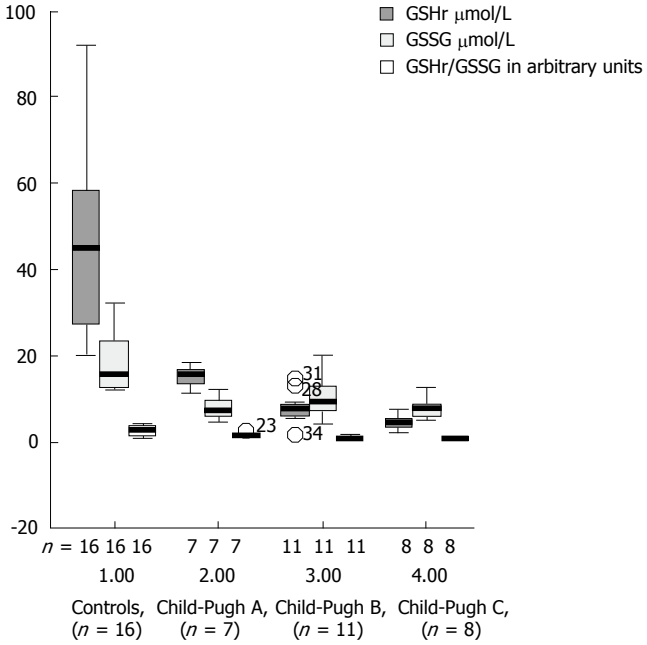


Figure 6 Boxes show the interquartile ranges. Vertical lines show 80% of the values (10^{th}-90^{th} percentiles). The bold horizontal line represents the median. $^{b}P < 0.01$ between controls and patients with Child-Pugh A cirrhosis or between patients with Child-Pugh B cirrhosis or between Child-Pugh C cirrhosis.

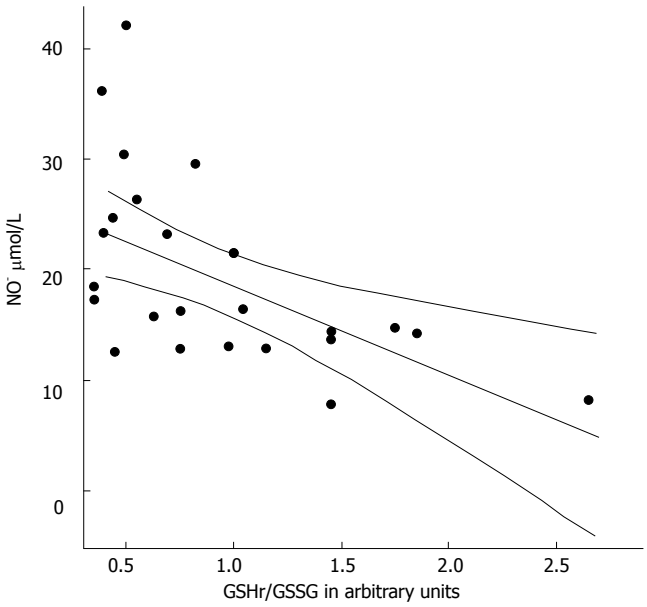


Figure 8 Correlation between NO· levels and GSHr/GSSG ratios. Linear regression with 95.00% mean prediction interval, $^{b}P < 0.01$; NO· μmol/L = 26.52 + -7.99; *R*-Square = 0.31.

in patients with alcoholic liver disease. Studies have shown increased plasma and tissue levels of markers of lipid peroxidation[13-16], and reduced hepatic and plasma antioxidant content[17-20].

In the liver, as in many other organs, NO· has many actions and cellular sources[4,5,16-21]. Determination of the levels of the NO· radical itself is hampered by its radical nature and very short half-life (in the range of seconds). Because NO· activates soluble guanylate cyclase to produce cyclic guanosine 3',5' monophosphate (cGMP) and stable nitric oxide metabolites, we measured the levels of its metabolites, NO_3^- and NO_2^-, in serum, and the levels of cGMP in plasma. The intracellular concentrations of ROS are tightly regulated by multiple defence mechanisms involving ROS-scavenging enzymes and anti-oxidant molecules, which include glutathione and GSH dependent enzymes. We also measured the levels of glutathione in its thiol-reduced (GSHr) and glutathione disulfide (GSSG) forms to better understand the defence mechanisms involving GSH operating in alcoholic cirrhotic patients[22-24].

The data of this study indicate that serum concentrations

of the NO• metabolites NO_3^- and NO_2^-, and those of plasma cGMP, an intracellular second messenger[25] of nitric oxide, in patients with alcoholic cirrhosis, were higher than those in healthy controls. The NO• and cGMP levels were higher in patients with decompensated liver cirrhosis (Child-Pugh B and C) than in those with compensated liver cirrhosis (Child-Pugh A) ($P < 0.001$). Our results also show a decrease in GSHt levels in alcoholic cirrhotic patients compared with healthy controls.

Associations were found between the levels of the biomarkers NO• and cGMP, as well as between the levels of each biomarker and MELD scores ($P < 0.01$). Significant negative correlations were observed between NO• levels and GSHr/GSSG ratios, and between MELD scores and GSHr/GSSG ratios, in the 26 alcoholic cirrhotic patients belonging to all three Child-Pugh classes ($P < 0.01$). The increased concentrations of GSSG in plasma provide a sensitive index of whole-body oxidative stress[26]. A deficiency of hepatic GSH and increased toxic free radical species may contribute to the progression of liver disease[17,18].

One of the more common free radicals produced in response to the ingestion of ethanol is oxygen in a deformed toxic state. These radicals, known as reactive oxygen species, include oxygen radicals and substances closely related to oxygen radical reactions. They can play a role in the development of alcoholic hepatitis by altering the redox state, damaging cell constituents in the liver and causing severe injury[27].

NO• can interact with ROS to form other reactive nitrogen species (RNS). These reactive nitrogen species secondarily promote important reactions such as nitrosation (production of nitrosamines), oxidation (mediating lipid peroxidation, DNA strand breaks and hydroxylation) or nitration (production of nitrotyrosines and nitroguanosines), leading to impaired cellular functions and enhanced inflammatory reactions[28]. On the other hand, ROS stimulates stellate cell activation and fibrogenesis[20,22,27]. Thus, oxidative stress seems to contribute to the pathogenesis of alcoholic liver injury and the progression of alcohol-induced liver disease to alcoholic cirrhosis[22,23].

Patients with advanced liver disease tend to develop a hyperdynamic circulation, which complicates cirrhosis. There is vast experimental evidence for enhanced NO• overproduction and associated cGMP formation in cases of portal hypertension. Moreover, several clinical studies have evaluated NO• synthesis and serum levels in various cirrhotic conditions[29-32]. Our study, including only alcoholic patients of different Child classes, strengthens previous investigations and adds further information with respect to the second messenger cGMP and its relationship with the redox state of GSH.

NO• or nitrite/nitrate measurements can be affected by creatinine levels, as demonstrated recently in acute decompensated cirrhotic patients[32]. In our clinical population, patients with Child C cirrhosis had elevated creatinine values compared to those with Child A cirrhosis, and this could also influence the results. A positive correlation was found between creatinine levels and NO• and cGMP mediators in all three Child-Pugh classes ($P < 0.01$, $P < 0.05$), but no correlation between these mediators and creatinine was found in either decompensated or compensated cirrhotic patients. However, we found an inverse relationship between GSHr/GSSG ratios and creatinine levels, only in decompensated cirrhotic patients ($P < 0.01$). The elevated mediator levels could also be caused by portal hypertension and hyperdynamic circulation where NO• is elevated and, therefore, have no relation to intrahepatic oxidative stress. Recent studies by Lluch *et al*[29] suggest that the serum concentration of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase is elevated in patients with alcoholic cirrhosis. This finding may offer an alternative compensatory mechanism to counteract the excessive visceral vasodilatation caused by excess production of NO• in patient with advanced liver disease.

In summary, our study shows that patients with decompensated alcoholic cirrhosis have higher levels of NO• and cGMP than compensated patients, and that there is a positive correlation between the clinical score of the disease (Child-Pugh classes and MELD scores) and the abnormalities observed. Our findings suggest that NO• and cGMP may contribute to progressive liver disease in these patients and influence the hemodynamic abnormalities. Our findings emphasize the need for further investigations correlating the serum levels of NO•, cGMP or GSH seen in decompensated cirrhotic patients with their clinical outcomes (for example, survival, variceal bleeding, renal failure and septic complications) to develop prognostic parameters and delineate new therapeutic strategies.

COMMENTS

Background

Alcohol accounts for 40%-50% of all deaths due to cirrhosis and remains the most common cause of liver-related mortality. Important pathophysiologic events that mediate alcohol-induced liver injury include increased oxidative stress, hepatocyte apoptosis and necrosis and deposition of collagen, with ensuing fibrosis.

Research frontiers

The correlation of mediators of oxidative stress with the severity of disease in patients with alcoholic liver injury has been studied intensively in recent years, mainly in patients with alcoholic hepatitis. We investigated patients with alcoholic cirrhosis and measured the levels of mediators of oxidative stress (namely, NO• and cGMP), antioxidant defences [thiol-reduced glutathione (GSHr) and glutathione disulfide (GSSG)] and correlated these levels with clinical liver disease severity.

Innovations

In contrast to previous studies, we measured the levels of not only NO•, but also cGMP (an intracellular second messenger of NO•) and correlated these with mediators of antioxidant defence. We found that the levels of nitric oxide metabolites and cGMP were high in alcoholic cirrhotic patients, particularly in those that were decompensated. There was a positive correlation between the clinical score of the disease (Child-Pugh classes and MELD scores) and the oxidative stress abnormalities observed.

Applications

Our findings suggest that NO• and cGMP may contribute to progressive liver disease in these patients. Further investigations correlating the serum levels of NO•, cGMP or GSHr seen in decompensated cirrhotic patients with their clinical outcomes (for example, survival, variceal bleeding, renal failure and septic complications) are needed to develop better prognostic parameters and delineate new therapeutic strategies.

Peer review

The authors measured nitric oxide metabolites in the serum and cGMP, GSH, and GSSG in the plasma with alcoholic cirrhosis patients. They found that nitric oxide metabolites and cGMP levels were high in alcoholic cirrhosis patients, particularly in decompensated alcoholic cirrhosis. Moreover, they found a significant negative correlation between GSH/GSSG ratio and MELD score in all cirrhotic patients.

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RAPID COMMUNICATION

Promoter polymorphism of transforming growth factor-β1 gene and ulcerative colitis

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Abstract

AIM: To elucidate the possible difference in two promoter polymorphisms of the transforming growth factor-β1 (TGF-β1) gene (-800G > A, -509C > T) between ulcerative colitis (UC) patients and normal subjects.

METHODS: A total of 155 patients with established ulcerative colitis and 139 normal subjects were selected as controls. Two single nucleotide polymorphisms within the promoter region of TGF-β1 gene (-509C > T and -800G > A) were genotyped using PCR-RFLP.

RESULTS: There was a statistically significant difference in genotype and allele frequency distributions between UC patients and controls for the -800G > A polymorphism of the TGF-β1 gene ($P < 0.05$). The frequency of the TGF-β1 gene polymorphism at position -800 showed that the AA genotype and the allele A frequencies significantly differed between the patients and healthy controls ($P < 0.05$). At position -509, there was no statically significant difference in genotype and allele frequency between the patients and control subjects.

CONCLUSION: The results of our study indicate that there is a significant difference in both allele and genotype frequency at position -800G > A of TGF-β1 gene promoter between Iranian patients with UC and normal subjects.

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Key words: Transforming growth factor-β1; Ulcerative colitis; Promoter; Polymorphism; Iran

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INTRODUCTION

Transforming growth factor-β1 is a cytokine produced by both immune and non immune cells, and it exhibits a broad range of functions. TGF-β1 controls the differentiation, proliferation, and state of activation of all immune cells, wound healing, angiogenesis and is implicated in immune abnormalities linked to cancer, autoimmunity, opportunistic infections, and fibrotic complications[1-5]. It has chemotactic properties and may stimulate cells to produce cytokines such as IL-1, IL6, and TNF-α at the sites of inflammation[1,6]. It is interesting that TGF-β1 plays an important role in promoting and activating extracellular matrix and bone remodeling. Paradoxically it is also involved in several immune suppressive mechanisms particularly as an inhibitor of intestinal epithelial proliferation[7-9]. It is proposed that the TGF-β1 production is under genetic control[7,10]. In the human TGF-β1 gene, which is located on chromosome 19q13 [11-15], eight polymorphisms are presently known. Three of them are localized in the promoter region at positions -988C > A, -800G > A and -509C > T from the first transcribed nucleotide[13].

Polymorphism of TGF-β1 has been investigated in several diseases. For instance, our own investigation indicates that polymorphism of this cytokine is not associated with the repeated spontaneous abortion[16,17]. Polymorphism of TGF-β1 and the risk of hepatocellular carcinoma have been investigated in patients with chronic hepatitis B virus infection[18,19]. In patients with liver cirrhosis, an association has been found between codon 10 and morphology of hepatocellular carcinoma in Korean population[20].

Inflammatory bowel disease is thought to result from inappropriate and ongoing activation of the mucosal immune system due to the presence of normal luminal flora. This aberrant response is most likely facilitated by defects in both the barrier function of the intestinal epithelium and the mucosal immune system[21,22]. As only two previous studies are available on the association of the -509 C > T TGF-β1 polymorphism with inflammatory

bowel disease, especially Crohn's disease[23,24], we investigated whether these two polymorphisms (-509, -800) are associated in south Iranian patients with established UC.

MATERIALS AND METHODS

Subjects

The subjects in this study were comprised of 155 unrelated patients with UC (69 males and 86 females, aged 23-51 years, mean 36.4 years) attending the Department of Gastroenterology, Namazi and Saadi Academic Hospitals of Shiraz Medical University. Disease duration was at least 2 years. Patients were asked about acute phases of the disease after diagnosis. Every patient chart was revisited to complete all available information. A total of 139 age- and sex- matched health volunteers with no history of chronic bloody diarrhea and abdominal pain (50 males, 89 females) served as controls (aged 18-61 years, mean 35.5 years). Diagnosis of UC was established on the basis of conventional endoscopic and histological criteria. Since UC is a dynamic disease and patients can fluctuate into a different phenotype during the course of the disease, we analyzed the clinical records of our patients at two time points: when patients visited the hospital and for the first time he/she was followed up (mean time after diagnosis of 1.5 years). All patients were sub-classified according to gender, age of onset, localization of the disease, need for steroid therapy, and emergent surgical treatment. In UC patients, the localization of gut involvement was defined as proctitis, left-sided (up to the splenic flexure) or pancolitis (beyond the splenic flexure).

DNA extraction and TGF-β1 genotyping

Venous blood was collected into EDTA-coated tubes. DNA was extracted from whole blood using the salting-out method. The following primers (MOLBIOL, Germany) were used for amplification of promoter regions -509 and -800. The sequences of PCR primers for the -509C/T and -800G/A polymorphisms are 5'-CAGTAAATGTATGGGGTCGCAG-3' (forward) and 5'-GGTGTCAGTGGGAGGAGGG-3' (reverse), and 5'-ACAGTTGGCACGGGCTTTCG-3' (forward) and 5'-TCAACACCCTGCGACCCCAT-3' (reverse) respectively. The PCR product sizes from these primers were 153 bp and 388 bp, respectively. PCR was performed in a total volume of 20 μL containing 100 ng genomic DNA, 20 pmol/L of each primer, 0.2 mmol/L dNTPs, 20 mmol/L Tris-HCl (pH 8.8), 10 mmol/L $MgCl_2$, and 1 unit of Taq polymerase (New England BioLabs Ipswich, MA). The PCR cycle conditions consisted of an initial denaturation at 94℃ for 3 min followed by 35 cycles at 94℃ for 60 s, at 61℃ for 60 s, at 72℃ for 60 s for -509 C/T, and at 94℃ for 45 s, at 61℃ for 45 s, at 72℃ for 45 s for -800G/A, and a final elongation at 72℃ for 5 min.

Genotyping was performed by restriction fragment length polymorphism (RFLP) analysis. The following restriction enzymes (Fermantes, Lithuania) were used for the digestion of amplified PCR products. For digestion of PCR products containing positions -800, *Nmu*CI and -509, *Eco811* was applied. The digested PCR products were resolved on 2% agarose gel and stained with ethidium bromide for visualization under UV light.

Table 1 Distribution of TGF-β1 genotype and alleles in UC patients and controls

TGF-β1 genotype and alleles	UC patients *n* = 155 (%)	Controls *n* = 139 (%)	*P* value
Genotype -800			
GG	125 (80.6)	104 (74.8)	< 0.05
GA	29 (18.8)	27 (19.4)	
AA	1 (0.6)	8 (5.8)	
Allele frequency			
G	279 (90)	235 (84.5)	< 0.05
A	31 (10)	43 (15.5)	
Genotype-509			
CC	57 (36.8)	56 (40.3)	
CT	65 (41.9)	63 (45.3)	0.3
TT	33 (21.3)	20 (14.4)	
Allele frequency			
C	179 (57.75)	175 (62.9)	0.19
T	131 (42.25)	103 (37.1)	

Statistical analysis

Allele frequencies at each polymorphic site were calculated by allele counting method. Deviation of the genotype counts from the Hardy-Weinberg equilibrium was tested using χ^2 test with 1 degree of freedom. Differences in the allele frequencies and genotype distributions between the patients with UC and the controls were analyzed by χ^2 or Fisher's exact tests when necessary.

RESULTS

Position -800 (G/A)

In this investigation, the change at position -800G > A of the TGF-β1 gene was studied using PCR-RFLP in 155 cases of UC and 139 normal Iranian subjects. At this position, homozygote GG was found in 125 (80.6%) UC patients and 104 (74.8%) normal subjects, heterozygote GA was observed in 29 (18.8%) UC patients and 27 (19.4%) normal subjects, while homozygote AA was demonstrated in only one of the patients, homozygote AA was shown in eight normal individuals. There was a statistically significant difference in genotype and allele frequency distributions between UC patients and controls for the -800G > A polymorphism of the TGF-β1 gene ($P < 0.05$) (Table 1). A typical genotyping at this position is represented in Figure 1A.

Position -509 (G/A)

In addition, the genotype at position -509C > T of the TGF-β1 gene was studied in the same study groups. At this position, homozygote CC was found in 57 (36.8%) UC patients and 56 (40.3%) normal subjects, heterozygote CT was observed in 65 (41.9%) UC patients and 63 (45.3%) normal subjects, homozygote TT was demonstrated in 33 (21.3%) UC patients and 20 (14.4%) normal subjects. There was no statistically significant difference in genotype distribution and allele frequency at this position between

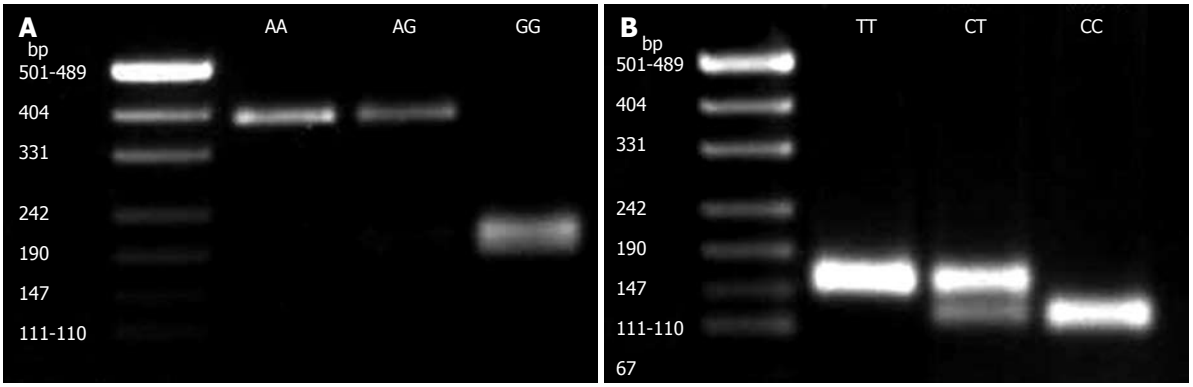


Figure 1 **A**: PCR-RFLP genotyping at position -800 (G/A). PCR product (388 bp) was digested with NmuCI restriction enzyme. From left to right: Lane 1: DNA size marker; lane 2: Homozygote AA; lane 3: Heterozygote AG; lane 4: Homozygote GG; **B**: PCR-RFLP genotyping at position −509 (C/T). PCR product (153 bp) was digested with Eco811 restriction enzyme. Lane 1: DNA size marker; lane 2: Homozygote TT; lane 3: Heterozygote CT; lane 4: Homozygote CC.

UC patients and controls. The distribution of TGF-β1 genotype and allele frequency in UC patients and controls are summarized in Table 1. A typical genotyping at position -509C > T is represented in Figure 1B. A Hardy-Weinberg equilibrium test was performed for the two polymorphisms.

Relation between other factors and polymorphisms

In our study, no significant relation was found between the two polymorphisms and factors such as gender, age of onset, cumulative dose of steroids, need for steroid therapy, localization of the disease, and need for emergent surgical treatment.

DISCUSSION

TGF-β1 is an immunoregulatory and immunosuppressive cytokine. Its role and function have been extensively investigated in inflammatory type of diseases[14]. It has been known that TGF-β1 has various biological and immunological functions, such as decreasing expression of MHC-Ⅱ, reducing synthesis of TH1/TH2 cytokine and suppressing the activation of both T and B lymphocytes. Plasma levels of TGF-β1 have been detected in several inflammatory and malignant diseases and the consensus is that the synthesis of this immunosuppressive cytokine is under the control of genetic factors[3,6,11]. TGF-β1 gene is located on chromosome 19 and contains 7 exons.

Several polymorphisms have been reported in TGF-β1 gene at positions -988, -800 and -509 located in the gene promoter[13-21]. Garcia-Gonzalez and co-workers showed that codon 10 and 25 TGF-β1 polymorphisms in Dutch IBD patients and healthy controls do not participate in defining the susceptibility to and tile nature of the clinical course in IBD[24]. Polonikov AV *et al*[28] reported that TGF-β1 gene polymorphism plays a role in the pathogenesis of gastric ulcer disease. Su Zg and colleagues[16] reported that allele A at position -800 and allele T at position -509 of TGF-β1 gene are increased in Chinese population with chronic obstructive pulmonary disease. Schulte CM[23] showed that IBD susceptibility is not associated with genetic variations in TGF-β1 promoter polymorphism.

In addition to the reports on genetic variations in TGF-β1 polymorphism in IBD, several studies showed that serum level of TGF-β1[17] and mRNA expression[18] are significantly increased in IBD patients.

In the present study, the genotype distribution and allele frequencies of polymorphisms at position -509C > T were not significantly different between UC patients and controls, which is consistent with the previous reports[24,30]. However, at position -800G > A both allele and genotype frequencies were significantly different between UC patients and controls. It has been reported that -800G > A substitution is thought to disrupt a consensus half-site for binding to the nuclear transcription factor (CRE-binding protein), consequently contributing to a lower production of total TGF-β1 in the circulation[7,26].

In our study, AA genotype was reduced in patients with IBD compared with controls. A similar finding was seen in UC patients and controls when the frequency of allele A was analyzed. These findings indicate that individuals bearing allele A and genotype AA are less susceptible to IBD than those lacking allele A and genotype AA.

To the best of our knowledge, polymorphism at position -800 of TGF-β1 gene promoter has not yet been reported in IBD patients. However, both plasma level of TGF-β1 and mRNA expression should be further studied and compared in IBD patients carrying genotype AA at this position.

There are a number of other polymorphisms with known influence on different TGF-β1 expressions[3,4]. To study the role of TGF-β1 haplotype in the pathogenesis of IBD, analysis of other known TGF-β1 gene polymorphisms will strength our current observation.

In conclusion, there is no significant difference in TGF-β1 polymorphisms at position -509 C > T and allele A is significantly associated with genotype AA at position -800 of the promoter region between southern Iranian UC patients and healthy individuals.

COMMENTS

Background

Inflammatory bowel disease (IBD) is a common disorder in our population with an increasing incidence but no clear cut etiology. Cytokines and their receptors,

immunogenetics and host -related factors are all involved in the disease susceptibility. TGF-β1 is produced by regulatory T lymphocytes with a profound suppressive effect on the induction phase of immune response. Plasma levels of TGF-β1 and its local synthesis, in addition to the contribution of genes encoding TGF-β1 have widely been investigated in IBD patients with no convincing and solid ground so far.

Research frontiers

Investigation of cytokine gene polymorphism in health and disease conditions has provided valuable information on the incidence of autoimmune diseases in different ethnics. Current data on TGF-β1 gene polymorphism in IBD are controversial due to the heterogeneity in clinical presentations of the disease and different methods. There is no evidence that TGF-β1 gene polymorphism is associated with IBD in European Caucasian population. Here we report the impact of allele A and genotype AA at -800 of TGF-β1 promoter region and susceptibility to Iranian patients with IBD.

Publications

Schults *et al* and Garcia Gonzalez reported that polymorphism at promoter region has no association with IBD in European individuals. Our data give another view on the significance of polymorphism of TGF-β1 promoter region in IBD patients.

Innovations and breakthroughs

Our findings provide a window for investigation on the mRNA and local secretion of TGF-β1 in IBD patients carrying allele A and genotype AA of TGF-β1 at position -800 of promoter region.

Application

By extending this investigation in a larger sample size within other ethnic groups or in a broader study in a format of meta-analysis, a genetic marker for screening of individual susceptible to IBD can be expected.

Peer review

This is a very interesting and original paper that merits to be fully published without any substantial modifications.It is also in good english written.

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RAPID COMMUNICATION

Prevalence and dietetic management of mild gastrointestinal disorders in milk-fed infants

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Abstract

AIM: To assess the prevalence of mild gastrointestinal disorders in milk-fed infants in paediatric practice, and to evaluate the effectiveness and satisfaction with dietetic treatment.

METHODS: A cross-sectional epidemiological study was first carried out. A total of 285 paediatricians included 3487 children seen during a period of one week. In a second phase an observational, prospective and multicentre study was conducted and 2069 milk-fed infants with mild gastrointestinal disorders (colic, constipation, regurgitation and diarrhoea) were included. There was a baseline visit (start of treatment) and a final visit four weeks later. The effectiveness of the various Novalac formulas, as well as the satisfaction of the parents/tutors and paediatricians with the dietetic treatment were assessed at the final visit.

RESULTS: The prevalence of mild gastrointestinal disorders was 27.8% of all paediatrician consultations (9.2%, 7.8%, 6.1% and 4.6% in relation to colic, constipation, regurgitation and diarrhoea, respectively). The several Novalac adapted milk formulas resolved 88.4% of the mild gastrointestinal disorders. Depending on the type of disorder, differences in response rate were observed. The highest effectiveness was recorded with respect to diarrhoea (92.6%), followed by constipation (91.6%), colic (87.6%) and regurgitation (81%). Overall, 91% of the paediatricians and 88.8% of the parents/tutors were satisfied or very satisfied with the Novalac adapted milk formulas.

CONCLUSION: Mild gastrointestinal disorders show a high prevalence in paediatric practice. The Novalac adapted milk formulas have been shown to be effective in treating mild gastrointestinal disorders in milk-fed infants in the context of routine clinical practice.

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Key words: Mild gastrointestinal disorders; Prevalence; Adapted infant formulas; Effectiveness; Satisfaction

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INTRODUCTION

Human milk is a complete and complex species-specific food that provides all the nutrients needed for the growth of infants born to term during the first 4-6 mo of life. There is total agreement that maternal milk is the feeding method of choice for infants. However, at present, 25% of our population does not breastfeed, while 50% of women do so for only a reduced period of time (3-4 mo). In the event of contraindication or refusal to breastfeed, artificial nursing measures must be adopted. In many of these cases the infant may suffer mild gastrointestinal disorders (MGDs) during the period of lactation[1] including colic[2], regurgitation[3], diarrhoea[4] and constipation[5]. These symptoms cause discomfort for the infant and are a source of concern for parents. Acute diarrhoea cannot be considered a dysfunction as such; however, since it can be treated by dietetic measures, it has been included among these MGDs.

Interest among paediatricians to solve these disorders and improve knowledge of their physiopathology has led the infant food industry to develop specific formulas for these conditions. In many cases, dietetic adjustments are the first link in the treatment chain. At present, a broad range of artificial formulas have been developed[6] for feeding "healthy" infants. These formulas can partially or completely replace human milk, covering the normal nutritional requirements of the infant. In the case of infants with MGDs, adapted formulas have been developed[7,8] in which some of the nutrients have been specifically modified with the purpose of minimizing or resolving the digestive disorder while ensuring optimum infant growth. These formulas are manufactured according to the recommendations of the European Society for

Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)[9-11], the European Commission[12,13], and the Royal Decrees of the Spanish Ministry of Health[14,15].

The present study investigates the prevalence of MGDs in Spanish infants under four months of age and seen in paediatric practice. An evaluation is also made of the effectiveness of the specifically elaborated formulas belonging to the Novalac line of products (United Pharmaceuticals, France; Chiesi España S. A, Spain) [Novalac Anti-Colic (low lactose, adapted formula), Novalac Anti-Regurgitation (thickened with starch and enriched amylopectins), Novalac Anti-Diarrhoea (lactose-free and with adapted concentration of electrolytes), or Novalac Anti-Constipation (adapted concentration of magnesium and lactose)], in relation to the number of avoided episodes of colic, regurgitation, diarrhoea and constipation among infants exclusively fed with artificial milk formulas. Lastly, satisfaction with the Novalac line of products among both parents/tutors and paediatricians, was evaluated along with its relation to resolution or reduction of the symptoms associated with MGDs.

MATERIALS AND METHODS

Study design and patients

The present study comprises of two sub-studies. The first sub-study is a cross-sectional data-collecting study involving a 7-d period to estimate the prevalence of infants less than four months of age with MGDs such as colic, constipation, regurgitation and diarrhoea, among the global infant population seen in paediatric practice for any reason. The second sub-study in turn corresponds to a prospective longitudinal survey of the effectiveness of dietetic treatment with the Novalac range of formulas specifically developed for such MGDs.

A total of 285 paediatricians throughout Spain (except Ceuta and Melilla) participated in the study, under real-world conditions of clinical practice. Representation of the nursing infant population by Spanish Autonomous Communities was maintained. To investigate the prevalence of infants with MGDs in paediatric practice, each investigator included all infants less than four months of age seen in the clinic for any reason, during a period of one week. The infants of the cross-sectional study presenting MGDs with artificial milk formulas, and who according to medical criterion could be fed with the Novalac range of formulas, were enrolled in the longitudinal study. During one week, each investigator consecutively included 10 infants that met the selection criteria. This period was prolonged for two weeks until the full quota designated to each investigator was covered.

The selection criteria were: (1) infants up to four months of age fed with artificial milk formulas; (2) the presence of MGDs; (3) the possibility of feeding the infants with some product of the Novalac line of formulas; and (4) continuation of these formulas on an exclusive basis for at least 30 d (with no incorporation of other foods to the diet).

The cross-sectional study included 3487 infants to assess the prevalence of MGDs. The prospective study for evaluating the efficacy of the Novalac line of products involved 2069 patients with MGDs. The patients were visited on two occasions: at the time of inclusion and after four weeks. In the case of infants with diarrhoea, the final visit took place after 4-7 d. A total of 1441 infants completed follow-up.

Study variables

During the initial visit, data were collected relating to sociodemographic (age and sex) and clinical variables (gestational age, weight and height at birth and at the time of the visit, and the type and duration of the disorder). A questionnaire addressing the different symptoms and their intensity was designed for each disorder: (1) Constipation: number of stools/d, consistency, irritability, need for external help to defecate; (2) Regurgitations-vomiting: number/d, duration of the feedings, volume ingested; (3) Colic: excess gases, duration of crying, duration of bottle feeding; (4) Diarrhoea: number of stools and their characteristics.

The effectiveness of the dietetic treatment was evaluated on the final visit. Anthropometric data were collected (weight, height), along with the number of days to disappearance or improvement of the disorder, the evolution of symptoms, adverse events, and satisfaction of the parents/tutors and paediatricians with the treatment. Also documented was paediatrician contact after 15 d with the parents by telephone, to know the condition of the infant (in those cases where the paediatrician considered the call relevant).

The satisfaction of the parents/tutors and paediatrician with the Novalac formulas used for the MGDs was assessed on occasion of the final visit by means of a Likert-type scale with five possible answers (from very satisfied to very dissatisfied).

Statistical analysis

Duplicate data introduction was carried out, with validation of the database. Analysis of the prevalence of MGDs was based on a descriptive analysis of the sociodemographic and clinical characteristics of the study population. Continuous variables were reported as the mean and standard deviation (SD), while categorical variables were expressed as the number and percentage of infants per response category. The description of the characteristics of the infants was made for the total study sample, with stratification according to MGD presence.

A descriptive analysis was made of the number and percentage of infants seen in paediatric practice for MGDs, calculating the confidence interval (CI) of prevalence with a level of significance of 0.05. In order to assess variability in the Spanish setting, comparative studies were made of the prevalence of MGDs among the different regions in the country, based on the chi-square test.

Calculation was made of the number of days needed for disappearance or improvement of the disorder, along with the mean number (95% confidence interval) of days of MGD persistence.

A descriptive analysis was made of satisfaction as expressed by the parents/tutors and paediatricians. To this effect we used descriptive indicators such as the mean,

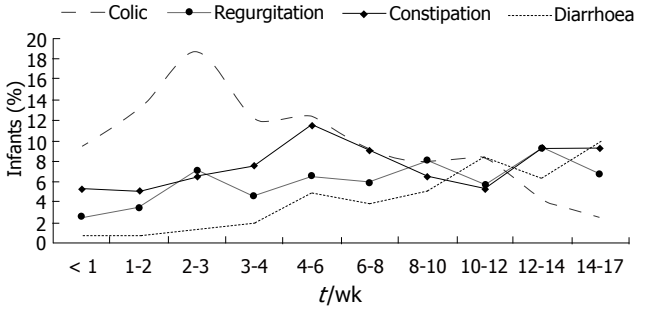


Figure 1 Percentage presentation of mild gastrointestinal disorders (MGDs) according to infant age and mean age at presentation.

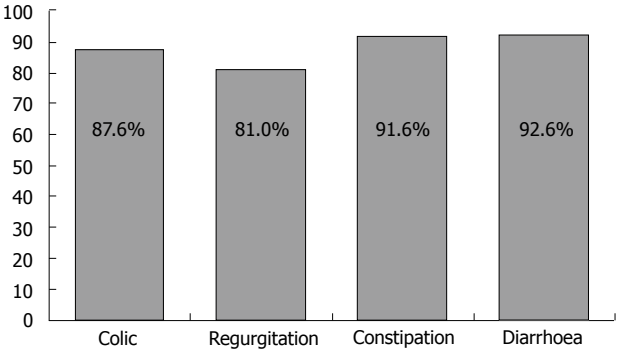


Figure 3 Percentage resolution of disorders with the Novalac formulas.

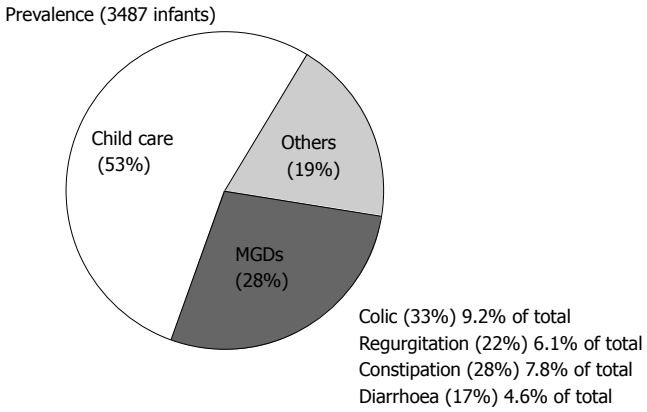


Figure 2 Prevalence of mild gastrointestinal disorders (MGDs) in paediatric practice.

standard deviation, median and percentiles 25 and 75. A study was made of the relationship between parent/tutor satisfaction with the Novalac line of products and their effectiveness, based on the Student t-test for independent data.

The SAS version 8.02 statistical package for Microsoft Windows was used throughout. In all the statistical tests a level of significance of $\alpha = 0.05$ was used.

RESULTS

The results of the cross-sectional study were obtained from the infant population included in the period of one week commented above, and involved 3487 infants. Boys predominated slightly over girls (52.2% *vs* 47.8%). Patient age at consultation was between under one week and 17 wk. Colic manifested at earlier ages (6.2 wk on average), followed by constipation (7.6 wk), regurgitation (8.6 wk) and diarrhoea (10.4 wk) (Figure 1). Gestational age was 39 wk on average (SD: 1.6 wk), with a mean body weight at birth of 3.2 (SD 0.5) kg and a height of 49.5 (SD: 2.3) cm.

The prevalence of MGDs was 27.8% (95% CI: 26.3-29.2); of these disorders, 9.2% (95% CI: 8.3-10.2) corresponded to colic, 7.8% (95% CI: 6.9-8.7) to constipation, 6.1% (95% CI: 5.3-6.9) to regurgitation -vomiting, and 4.6% (95% CI: 3.9-5.3) to diarrhoea. Only 0.6% of the infants presented with other digestive disorders (anorexia/hyporexia and eating disorders), while 19.0% (95% CI: 17.8-20.4) were diagnosed with some other non-digestive problem (upper airways infection, bronchitis and dermatitis). Of the study series, 52.6% consulted for aspects related to child care (Figure 2). The prevalence considering only the 27.8% of infants with MGDs was: colic 31.2% (95% CI: 30.3-36.2), constipation 29.2% (95% CI: 25.3-30.9), regurgitation 21.3% (95% CI: 19.5-24.7) and diarrhoea 18.3% (95% CI: 14.2-18.9).

The longitudinal study included 2069 infants with MGDs: 646 infants with colic, 441 with regurgitation, 604 with constipation and 378 with diarrhoea. The effectiveness of dietetic treatment for these disorders was evaluated among the 1441 infants that completed follow-up. Premature study termination among some infants was due to adverse events in 2.7% of cases, parent decision in 6.9%, loss to follow-up in 1.64%, protocol violations in 2.46%, and non-specified reasons in 16.62%.

Effectiveness in resolving the different disorders, after feeding with the specific Novalac formulas under conditions of routine clinical practice, is reported in Table 1 and Figure 3.

Colic was resolved among 87.6% of the infants after a median of 8 d - the proportion of infants with normal bottle feeding increasing from 33.9% at baseline to 69.2%. Likewise, a considerable reduction was observed in excess gases (from 82.9% at baseline to 25.8%). The duration of crying decreased in all infants.

Regurgitation was resolved in 81% of the infants after a median of 6 d of follow-up. The figure decreased from six regurgitations and/or vomiting episodes a day to only two, and the time required for bottle feeding decreased 53% - with an increase in the volume of daily ingestions in 61.9% of the infants.

A full 91.6% of the cases of constipation were resolved within 7 d. The number of daily stools among the infants with constipation increased from 0.6 (SD 0.7) stools/d to 1.7 (SD 0.8) stools/d. At the end of follow-up, the stools were found to be normal in 95.6% of the infants, while 89.6% presented no pain or discomfort, and 91.2% required no external help in defecating.

The infants with diarrhoea in turn, showed a decrease (mean [SD]) from 5.4 (1.8) to 2.2 (1) stools/d. Fever was absent in 98.5% of the cases, and 79.7% of the infants showed normal defecation at the end of treatment. A full 92.6% of the cases of diarrhoea were resolved within three days (Figure 4).

Development of all infants fed with the Novalac

Table 1 Description of clinical characteristics of patients at baseline and at the end of the study

		Baseline	30 d
Colic	Excess gases	82.90%	25.80%
	Duration of crying		
	< 1 h/d	12.10%	85.20%
	1-3 h/d	53.00%	13.30%
	> 3 h/d	34.80%	1.60%
Regurgitations	Regurgitation and/or vomiting per day [mean (SD)]	6.9 (4.5)	2.4 (1.8)
	Mean number of daily ingestions	6.0	5.6
	Duration of daily ingestions		
	Increases	-	24.50%
	Without change	-	22.40%
	Decreases	-	53.10%
	Volume of daily ingestion		
	Increases	-	61.90%
	Without change	-	18.70%
	Decreases	-	19.40%
Constipation	Type of stools		
	Normal	33.40%	95.60%
	Hard	66.60%	4.40%
	Presence of pain or discomfort		
	Yes	90.00%	10.40%
	No	10.00%	89.60%
	External help needed for defecation		
	Yes	76.10%	8.80%
	No	23.90%	91.20%
		Baseline	7 d
Diarrhoea	Presence of fever		
	Yes	21.40%	1.50%
	No	78.60%	98.50%
	Number of stools per day [mean (SD)]	5.4 (1.8)	2.2 (1)
	Type of stools		
	With mucus and/or blood	5.20%	1.10%
	Liquid	45.00%	4.80%
	Semiliquid	49.80%	14.40%
	Normaly	0.00%	79.70%

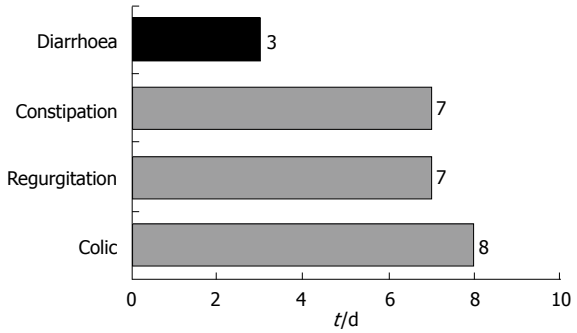


Figure 4 Mean days to resolution of mild gastrointestinal disorders (MGDs).

formulas was within the normal range.

Ninety-one percent (91%) of the paediatricians were satisfied or very satisfied with the dietetic regimen, though statistically significant differences were observed between groups ($P < 0.001$) - increased satisfaction being recorded among the paediatricians that treated diarrhoea and constipation versus those that treated colic and regurgitations. The greatest levels of satisfaction corresponded to the treatment of diarrhoea, with 95.9% of the paediatricians claiming to be satisfied, followed by constipation (92.8%), colic (88.5%) and regurgitation (87.8%).

As to parent satisfaction with the treatment ($P < 0.001$), 88.8% claimed to be satisfied with the course of the disorder - the specific figures being 93.7% in the case of diarrhoea, followed by constipation (90.0%), colic (88.1%) and regurgitation (83.3%).

Regarding the adverse events recorded during the study, only 3.9% of the infants receiving Novalac formulas suffered some adverse event. The most frequent affected the digestive tract (1.4%), including diarrhoea and constipation, and respiratory apparatus (0.7%) (e.g. bronchitis and bronchiolitis). Ten infants (0.5%) required hospital admission for different reasons: septicaemia ($n = 1$), dehydration ($n = 2$), vomiting ($n = 1$), hernia ($n = 1$) and bronchitis or bronchiolitis ($n = 2$).

DISCUSSION

Maternal breast milk is the usual food source for infants, though when breastfeeding is not possible, artificial lactation can be provided in the form of starting formulas, with the introduction of follow-up formulas 4-6 mo later, to ensure optimum nutritional development. During this period mild gastrointestinal disorders (MGDs) are not uncommon, and may be treated with dietetic measures.

This study for the first time provides information on the prevalence of these disorders in Spain, and on

the efficacy of dietetic treatment using the different formulas of the Novalac product range. The results of the prevalence study indicate that colic in infants is the MGD most often seen in paediatric clinics (9.2%), representing 33.3% of all MGDs.

Infant "colic" is an ill-defined term that is often used in reference to different situations found in paediatric practice[16]. Due to the lack of a clear definition, prevalence and treatment studies characteristically include heterogeneous groups of infants. These are usually healthy children that in the first four months of life develop paroxysmal crying without any apparent cause, and that gradually subsides. The underlying aetiology has not been clearly established, though the disorder has been related to alterations in motility[8], prostaglandin dysfunction, abnormal serotonin concentrations, and neuropeptide immaturity. Other authors more inclined to seek behavioural explanations tend to relate infant colic to alterations in the family environment, or even consider it a "normal" form of behaviour in infants with a more irritable temperament.

Human milk contains 7 g of lactose per 100 mL. However, in the first few weeks of life, infants present physiological or functional lactase insufficiency that limits absorption of these amounts. The non-hydrolyzed lactose reaches the colon, where it ferments to yield lactic acid, short-chain fatty acids, methane, carbon dioxide and hydrogen. This exerts a beneficial effect in that the stools become semiliquid as a result. However, is some cases such fermentation gives rise to excess gas[17-19], which favours the development of colic. In effect, different studies have related excess crying with excess intestinal gas[20].

The Novalac Anti-Colic formula offers an important reduction of the symptoms. Colic was resolved in 87% of the cases within one week, with a reduction in crying time, improvement in feeding continuity, and a reduction in the amount of intestinal gas.

In this study, constipation was the cause of consultation in 7.8% of the infants (representing 28% of all MGDs). A recent article published by Quinlan *et al*[21] studied the stool characteristics of 844 infants between 7-15 d old. Hard stools were recorded in 17% of the formula-fed infants, and in none of the breastfed infants. Breastfeeding entails a series of compensating physiological mechanisms that avoid constipation[22]. The Novalac Anti-Constipation formula bases its therapeutic efficacy partially on its high lactose content (8.1 mg/100 mL). The non-hydrolyzed lactose reaches the colon, where it is metabolized by the anaerobic flora, producing an osmotic laxative effect, since it attracts water into the intestinal lumen[17-19]. The magnesium contained in the formula (within the authorized maximum limits: 9.1 mg/100 mL) also enhances the laxative effect due to its osmotic action, and stimulates bowel motility by inducing cholecystokinin (CCK) secretion[23-25]. The efficacy of such treatment is reflected by an increase in the number of stools, and a reduction in the number of hard depositions, associated discomfort and the need for external help in defecation.

The next most prevalent MGD in the present study was regurgitation (22% of the total MGDs). Regurgitation and vomiting are the final manifestation of reflux. The aetiology of reflux in infants is of a multifactorial nature, and has not yet been fully elucidated. Postprandial reflux is accepted to be physiological, and occasionally occurs in almost all infants. However, in some cases the clinical picture is particularly manifest and can produce important discomfort for both the child and family. It is generally agreed that uncomplicated gastro-oesophageal reflux should not receive pharmacological treatment (antacids, prokinetic agents, H2 receptor blockers), though effective measures such as postural and dietetic management are recommended. In any case, the same authors that initially recommended such treatment have recently questioned it[26].

Milk thickening is a therapeutic practice that has been recommended for decades. Increased food viscosity and density reduces the reflux rate but does not modify the rest of parameters indicative of pathological gastro-oesophageal reflux. The products used as thickeners for the most part have been locust bean flour, pectin and cellulose. Cereal starch (rice and corn) has also been proposed, and offers the advantage of avoiding the possible adverse effects associated with the presence of galactomannans[27]. The Novalac Anti-Regurgitation formula contains a specially selected corn starch (having high amylopectin content) in a proportion of 1.9 g per 100 mL. It is pregelatinised and contributes to the increased viscosity in the stomach (on average 10 fold the viscosity in the feeding bottle). The presence of medium-chain fatty acids in turn favours a reduction in gastric emptying[28]. The efficacy of the treatment is reflected by the results obtained in the present study. In effect, the number of regurgitations/vomiting episodes decreased during the 7 d of treatment, and improvements were also seen in the rapidity of bottle ingestion, with a larger ingested volume.

Lastly, acute gastroenteritis represented 16.5% of the global MGDs in our study. Acute gastroenteritis, of a bacterial or viral origin, is characterized by alterations in the normal displacement of water and electrolytes within the intestinal lumen. When diarrhoea is caused by a virus (fundamentally rotavirus) transient lactase deficiency may also result. The duration of this deficiency is usually 7-15 d, though infants that are malnourished or suffer serious intestinal lesions may have persistent diarrhoea for up to 18-24 mo. In those cases in which deficient lactose absorption is suspected, lactose-free formulas are justified[29,30]. In most cases a single week of lactose exclusion suffices, after which the usual formula is reintroduced, while monitoring tolerance.

The most recent developments point to the advisability of introducing soluble fibre[31]. The use of amylase-resistant carbohydrates is an innovation that takes advantage of the functional properties of the colon - specifically, the metabolic activity of the anaerobic flora and the absorption capacity of the colon mucosa. The purported mechanism involves use by the colon mucosa of the short-chain fatty acids produced by bacterial metabolism of non-absorbed carbohydrates, to favour the absorption of water and electrolytes[32]. In the colon, many carbohydrates are fermented by the anaerobic flora, resulting in short-chain fatty acids: propionate, acetate and butyrate. In the mucosa, these fatty acids stimulate the absorption of water and electrolytes.

The Novalac Anti-Diarrhoea formula contains no lactose, and moreover incorporates a series of novelties. The presence of electrolytes within the accepted maximum range (31 mg of Na/100 mL, 83 mg of K/100 mL and 49 mg of Cl/100 mL) ensures an increased mineral supply for those patients who after discontinuing oral rehydration remain at risk of excessive losses. On the other hand, the presence of pectin (1.5 g per 100 mL) offers the advantage versus other fructo-oligosaccharides of being totally fermented in the colon, since it is a larger and more viscous molecule - thereby favouring short-chain fatty acid production.

The present study for the first time offers information on the prevalence of mild gastrointestinal disorders in Spanish infants under four months of age seen in paediatric clinical practice. Dietetic intervention with the Novalac formulas has been shown to be effective in resolving these disorders in the routine clinical setting - with a significant reduction in associated symptoms. A close relationship has been found between satisfaction among the parents/tutors and paediatricians and the effectiveness of the dietetic treatment provided. In turn, the low prevalence of treatment-related adverse events reflects the good tolerability of the formulas belonging to the Novalac range of products.

COMMENTS

Background

Different milk-feed infant formulae have appeared in the market during the last decades for the nutritional treatment of different entities such as colic, constipation, regurgitation and acute diarrhoea, called "mild gastrointestinal disorders" (MGD), There are few data about the prevalence or these MGD and its evolution after the intake of nutritional formulae.

Research frontiers

This study investigated the MGD prevalence, MGD physiopathology, International Committees and Scientific Societies position in front of new formulations composition, as well as clinical response with these new formulae.

Innovations and breakthroughs

In this article we have published, for the first time in our country, relevant data regarding the MGD prevalence and dietetic clinical response in a huge number of milk-fed infants (*n* = 2069). Among international publications one can find lots of information about recommendations of how these formulations have been made and its usages indications and efficacy, but never in a population as big as this.

Applications

This study allows the real acknowledgment about MGD prevalence, and also explains why new formulae composition have to be used and its clinical response. It also increases paediatricians possibility of use of these formulations, giving them confidence regarding clinical efficacy and nutritional safety.

Terminology

Mild gastrointestinal disorders: colic, constipation, regurgitation and acute diarrhoea.

Peer review

The authors are to be congratulated on an important and well presented study.

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RAPID COMMUNICATION

Efficacy and safety of pegylated-interferon α-2a in hemodialysis patients with chronic hepatitis C

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Abstract

AIM: To evaluate the efficacy and safety of pegylated-interferon alpha-2a in hemodialysis patients with chronic hepatitis C.

METHODS: Thirty-six hemodialysis patients with chronic hepatitis C were enrolled in a controlled and prospective study. All patients were treatment naive, positive tested for anti-HCV antibodies, and positive tested for serum HCV-RNA. Twenty-two patients received 135 μg peglyated-interferon α-2a weekly for 48 wk (group A). The remaining patients were left untreated, eleven refused therapy, and three were not candidates for kidney transplantation and were allocated to the control group (group B). At the end of the treatment biochemical and virological response was evaluated, and 24 wk after completion of therapy sustained virological response (SVR) was assessed. Side effects were monitored.

RESULTS: Of 22 hemodialysis patients, 12 were male and 10 female, with a mean age of 35.2 ± 12.1 years. Virological end-of-treatment response was observed in 14 patients (82.4%) in group A and in one patient (7.1%) in group B (P = 0.001). Sustained virological response was observed in 11 patients (64.7%) in group A and in one patient in group B (7.1%). Biochemical response parameters normalized in 10/14 patients (71.4%) at the end of the treatment. ALT levels in group B were initially high in six patients and normalized in one of them (25%) at the end of the 48 wk. In five patients (22.7%) therapy had to be stopped at mo 4 due to complications of weakness, anemia, and bleeding.

CONCLUSION: SVR could be achieved in 64.7% of patients on hemodialysis with chronic hepatitis C by a treatment with peglyated-interferon α-2a. Group A had a significantly better efficacy compared to the control group B, but the side effects need to be concerned.

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Key words: Peglyated-interferon; Hemodialysis; Chronic hepatitis C; Treatment; Efficacy; Safety

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INTRODUCTION

Hemodialysis patients are at high risk of infection by hepatitis C virus (HCV) because the hemodialysis unit is a medical environment where exposure to blood is frequent. Therefore, the prevalence of HCV infection, from less than 5% to over 70% in some countries, is greater than the prevalence of HCV infection in the general population[1]. HCV infection is an important cause of morbidity and mortality among patients with end-stage renal disease (ESRD)[2]. HCV infection in patients on maintenance hemodialysis was reported in 10%-59% of patients, in comparison to 0.3%-1.5% observed in the general population[3]. The prevalence of HCV infection is 10%-20% in dialysis patients in developed countries[4,5] and much higher in less developed countries[6]. The prevalence of anti-HCV antibodies among dialysis patients was 40.3% in Turkey[7], 30% in India[8], and 43.9% in Saudi Arabia[9]. In United States of America in 2000, 8.4% of haemodialysis patients were anti-HCV positive[10].

The main mechanisms involved in nosocomial infection with HCV in haemodialysis patients are filter re-use, the use of contaminated haemodialysis machines, and contamination of medical staff's hands. It has been shown that the incidence of HCV infection in haemodialysis patients increases if the medical staff member does not change her/his gloves before injecting each patient and if hepatitis C patients undergo haemodialysis in the same room[11].

The eradication of HCV infection is thought to be valuable for patients with ESRD, especially those who are candidates for kidney transplantation[12]. To prevent the development of these complications and to make these patients suitable for transplantation, standard interferon-α

was used in various doses or regimes for the treatment of these patients[13].

The supplement of a polyethyleneglycol molecule to interferon produces a biologically active molecule with a longer half-life time and more favorable pharmacokinetics; these characteristics enable for a more appropriate once-weekly dosing. When pegylated-interferon α-2a (PEG-IFN) alone is given to chronic hepatitis C patients with normal renal function for 48 wk, the sustained virological response (SVR) rate is approximately twice that with standard interferon[14,15].

This study evaluated the tolerability and efficacy of PEG-IFN in patients with chronic hepatitis C. Therefore, we carried out a controlled prospective longitudinal study to assess the biochemical and the virological response at 48 wk of treatment with PEG-IFN and its tolerability in hemodialysis patients with chronic HCV infection.

MATERIALS AND METHODS

Study design and patients

The present controlled and prospective study was carried out in the Department of Infectious Diseases in Dicle University Hospital, and in one Private Dialysis Center in Diyarbakir, Turkey. In total, 58 among the 161 patients with total hemodialysis in this center were anti-HCV positive (36%). Of the 58 patients, 38 were HCV-RNA positive (65%). Two patients were excluded because they had decompensated liver disease (n = 1), coinfection with hepatitis B virus (n = 1), or because they were lost to follow-up. Thirty-six HCV-RNA positive patients were informed about the benefits and possible risks of PEG-IFN treatment. Fourteen patients were excluded from the study, eleven refused the therapy, and three were not candidates for kidney transplantation and were allocated to the control group (group B). The remaining 22 patients were allocated to the PEG-IFN treatment group (group A). All patients underwent chronic hemodialysis treatment for ESRD during the study period. Hemodialysis was carried out routinely 2-3 times weekly in the patient population. All patients were anti-HCV antibody positive and had detectable HCV-RNA by polymerase chain reaction for at least 6 mo. It has been reported that liver biopsy (histology) is not suggested in the patient with chronic hepatitis C and end-stage renal diseaese because of high bleeding risk.

Inclusion criteria

PEG-IFN therapy was performed in patients meeting the following inclusion criteria: (1) Age < 65 years; (2) absence of pregnancy and agreement to avoid pregnancy during therapy; (3) informed consent; (4) lack of autoimmune, thyroid, psychiatric, or malignant disorders; (5) negative HIV antibody test; and (6) thrombocyte count > 70 000/mm^3 and white blood cell count > 3000/mm^3.

Exclusion criteria

Patients meeting at least one of the following criteria were excluded: (1) Age < 18 or > 65 years; (2) presence of coinfection with HBV or HIV; (3) receiving immunosuppressive therapy or other treatments, namely antihistaminics, non-steroidal anti-inflammatory drugs, aciclovir, or amiodarone; (4) previous treatment for HCV infection; (5) alcohol consumption > 40 g/d; (6) active drug addiction; (7) evidence of hepatocellular carcinoma (α-fetoprotein > 100 ng/mL); (8) hemophilia; or (9) contraindication to interferon therapy.

Study protocol

Patients (group A) enrolled in the study received 135 μg PEG-IFN (40 kDa) (PEGASYS; F. Hoffmann-La Roche, Basel, Switzerland) weekly for 48 wk at the end of dialysis session. All treated patients were evaluated at the end of wk 12 of treatment. The antiviral treatment was continued if the patient had at least a 2-log decline from baseline HCV-RNA level. Patients were followed up and evaluated for 24 wk after completion of treatment. Therapy was monitored weekly by complete blood count and liver function tests (alanine aminotransferase [ALT; U/L], aspartate aminotransferase [AST; U/L]) for 3 mo, then monthly. HCV-RNA testing was carried out before treatment and then every 3 mo. Anti-HCV antibody was measured by a third generation commercial ELISA (Innotest HCV Ab IV; Innogenetics NV, Ghent, Belgium). Liver biopsy was not performed in hemodialysis patients. Serum HCV-RNA was quantified using a reverse transcriptase-polymerase chain reaction assay (Amplicor HCV ver. 2.0; Roche Diagnostic Systems, Branchburg, NJ) with a dynamic range being between 600 and 500 000 IU/mL. All samples were blindly tested in duplicate.

Virological and biochemical response criteria

In group A virological early response (virological EAR), virological end-of-treatment response (virological EOR), and sustained virological response (SVR) were defined as negative HCV-RNA by PCR at 12 and 48 wk of the therapy, and 6 mo after completion of therapy, respectively. In the treatment group, biochemical early response (biochemical EAR), biochemical end-of-treatment response (biochemical EOR), and sustained biochemical response (biochemical SR) were defined as the normalization of serum ALT activity at wk 12 and 48 and 6 mo after completion of therapy, respectively. Although group B patients did not receive PEG-IFN, biochemical and virological recovery at 12, 48, and 72 wk after the beginning of the study were categorized as early response (EAR), end-of-treatment response (EOR), and sustained response (SR), too.

Statistical analysis

Student's t test was used to compare mean values between groups, and the χ^2 test and Fisher's exact test were performed to analyze qualitative data. Parametric data are expressed as mean ± SD. A value of $P < 0.05$ was considered statistically significant. Statistical analysis was performed by using SPSS version 10.0 (SPSS Inc; Chicago, IL).

RESULTS

Enrollment started in November 2004 and the study was finished in July 2006. Seventeen of 22 patients finished therapy. The mean serum viral load before treatment was

Table 1 Demographic and clinical features of study patients

Variables	Group A (n = 22)	Group-B (n = 14)	P
Age (yr)	35.2 ± 12.1	37.1 ± 14.6	0.629
Male (%)	12 (54.5)	10 (71.4)	0.448
BMI (kg/m^2)	25.6 ± 3.3	26.1 ± 3.9	0.78
ALT (IU/L)-Range	59.2 ± 22.4-(33-109)	44.8 ± 20.9-(21-71)	0.489
Viral load (x 10^5 copy/mL)	7.9 ± 4.8	8.1 ± 4.5	0.89
Genotype 1b (%)	86.4	92.9	0.56
HD duration (mo)	52.4 ± 24.7	49.8 ± 21.1	0.95

Table 2 Virological response rates

	Group A (n = 22)	Group-B (n = 14)	P
Early response			
Virological (%)	82.4	0	< 0.001
End-of-treatment response			
Virological (%)	82.4	0	< 0.001
Sustained response			
Virological (%)	64.7	0	< 0.001

2.4 × 10^5 copy/mL. At the beginning of therapy, ALT levels were found to be elevated in fourteen patients (63.6%). In nine of these patients, ALT activity decreased to normal levels within 12 wk of treatment (biochemical EAR 64.3%). At the end of the treatment, four patients still had high ALT levels (biochemical EOR 71.4%). In this group, the mean serum ALT activity at initiation was 59.2 ± 22.4 IU/L (range, 33-109 IU/L). This significantly decreased to 29.9 ± 13.7 IU/L and 21.8 ± 10.9 IU/L at wk 12 (P = 0.017) and at the end of the treatment (P = 0.001), respectively. At the beginning of the study, ALT levels were high in six patients in group B. One of the patients' levels became normal at 12 wk resulting in a biochemical EOR of 16.7%. In the control group, the mean ALT level was 44.8 ± 20.9 IU/L at the beginning. This value declined to 33.8 ± 21.7 IU/L at wk 12 (P = 0.786) and 33.1 ± 18.9 IU/L at wk 48 (P = 0.760).

The mean pretreatment serum HCV-RNA levels were 7.9 ± 4.8 × 10^5 copy/mL and 8.1 ± 4.5 × 10^5 copy/mL in group A and group B, respectively (Table 1).

The viral load was statistically similar between the groups (P = 0.890). All patients treated with PEG-IFN showed at least a 2-log decline from baseline HCV-RNA level. But HCV-RNA became undetectable in 82.4% of the patients at wk 12 of therapy. Virological EOR and SVR occurred in 82.4% and 64.7% of the patients (Table 2). Virological EOR and SVR 0% of the control group.

Therapy with PEG-IFN was associated with a higher rate of virological response than the control group (P < 0.001). All of the subjects had genotype 1. In the treatment group, three patients had genosubtype 1a and 19 had genosubtype 1b. In group B one subject had genotype 1a, and 13 had genotype 1b. There was no significant difference between the groups with respect to genotype distribution (P = 0.560).

Most adverse events were mild to moderate in severity, and all adverse events were typical of those previously reported for PEG-IFN. The drug was suitably tolerated by patients. Flu-like syndrome, throbocytopenia, leucopenia, and anemia were the most frequent side-effects and were experienced in nine patients (53%). These side-effects included flu-like syndrome in eight (47%), fatigue in six (35%), anemia in four (23.5%), thrombocytopenia in three (17.6%), and leucopenia in three of them (17.6%). We had to stop the treatment in five patients (22.7%) in fourth month at the begin of the treatment due to complications (two of anemia, two of weakness, one of gastrointestinal bleeding). The side-effects led to discontinuation of the treatment in five patients. Seventeen of 22 patients finished the treatment in spite of side-effects due to PEG-IFN. No patient had a serious infection during the treatment period.

DISCUSSION

In patients with normal renal function, pegylation increases the size of the molecule, delays its clearance, and enhances the therapeutic effect of standard IFN. It is possible to hypothesize that, in patients with renal failure; the clearance of PEG-IFN would be even more delayed, resulting in higher serum levels of the drug and in a longer half-life time.

The results of this study confirm the efficacy and safety of PEG-IFN therapy in hemodialysis patients with chronic hepatitis C. Treatment for 48 wk with PEG-IFN resulted in sustained virologic responses in 64.7% of patients. HCV infection increases the risk of death in patients on chronic hemodialysis, along with hepatocellular carcinoma and liver cirrhosis[16]. Many controlled and uncontrolled trials have focused on the treatment of chronic hepatitis C patients on chronic haemodialysis with IFN therapy[17], because treatment with PEG-IFN is rarely recommended.

Fabrizi *et al* have found a mean SVR of 37% in chronic hepatitis C patients on dialysis after IFN therapy. Sustained biochemical and virological response rates in patients under classical IFN therapy were reported as 0%-67% and 15.8%-64%, respectively[6]. Sporea *et al* have found, in treatment of these patients with standard IFN the sustained biochemical response of 46.1% and sustained virological response of 38.4% respectively 6 mo after interferon treatment[17]. The promising results at the standard IFN therapy in chronic haemodialysis patients with chronic hepatitis C, have shown that viral clearance occurs in 27%-64% of patients after 12 mo of treatment with standard IFN[18,19].

Patients with end-stage renal disease and chronic hepatitis C might have severe chronic hepatitis despite normal serum liver enzyme activity[20]. In our study, serum ALT levels were normal in 36.4% of the patients at the beginning of the study. Similarly, Perez *et al* reported normal ALT levels in 49% of patients at the beginning of treatment[20]. In the treatment group in our study, serum ALT levels became normal in 71.4% of the patients by the end of the therapy, whereas 16.7% of the patients in the control group had a biochemical response of end of therapy. In contrast, the side-effects of IFN treatment are very important for the patients with ESRD. In several situations, the IFN treatment could not be continued in those patients. Liver biopsy was avoided because of the risk of bleeding in these patients[21].

Recently, a pharmacokinetic study was carried out with

PEG-IFN in subjects with various degrees of stable renal failure who were not yet dialysis dependent. Adsorption and distribution of PEG-IFN were similar in subjects with stable chronic renal impairment versus individuals with normal renal function[22]. The dose of 135 μg of PEG-IFN in patients with ESRD gave similar serum concentrations to a dose of 180 μg in patients with normal renal function. On the trials of PEG-IFN in patients with end-stage renal disease have been designed using weekly doses of 135 μg (as opposed to 180 μg)[7].

HCV genotype 1 is very common in Turkey. Similarly, all of the patients in the present study had genotype 1. Although the response to IFN treatment is not excellent in genotype 1, our results were outstanding[23]. Most of the patients in our study were infected with genotype 1b (86.4% group A, 92.9% group B).

Recently, Kokoglu *et al* reported the results of a controlled study in which PEG-IFN 135 μg/wk for 48 wk was used in hemodialysis patients with HCV infection. They found 83.4% virological EOR and 71.4% biochemical EOR[7]. Sporea *et al* reported on a 50% SVR in hemodialysis patients receiving PEG-IFN 180 μg/wk[24]. In another study, virological response was 40% with PEG-IFN. The difference in virological response could be related to the duration of treatment in the last study (24 wk) and the molecular weight of PEG-IFN (17 kDa)[25]. We found in our study 82.4% virological EOR and 71.4% biochemical EOR.

PEG-IFNs are likely to become a valuable addition for HCV therapy in ESRD when combined with reduced ribavirin doses. However, the pharmacokinetics and tolerability of PEG-IFN and ribavirin combination therapy need to be studied in prospective studies[26]. To date, PEG-IFN and ribavirin combination therapy is the treatment of choice for patients with HCV infection. Ribavirin is metabolized by the kidneys and its clearance reduces in patients with ESRD. High ribavirin serum levels markedly increase the risk of hemolytic anemia and the use of ribavirin in uremic patients, who are often already anemic, could cause severe and life-threatening anemia. Thus, a combination therapy with ribavirin is not an option for treatment of chronic HCV infection in hemodialysis patients[27].

We found in the present study 64.7% SVR and we can expect that in these subjects the use of PEG-IFN would lead to a higher rate of SVR than that observed with standard IFN, probably with a higher rate of adverse effects[11]. PEG-IFN therapy had a successful efficacy in the present study but tolerability was not perfectly. We had to stop the treatment in five patients (22.7%) due to complications (two of anemia, two of weakness, one of gastrointestinal bleeding).

In conclusion, the results of the present study show PEG-IFN (40 kDa) administered at a dose of 135 μg weekly for 48 mo was efficacious but not perfectly tolerable in dialysis patients with HCV infection.

COMMENTS

Background

Our study showed that PEG-IFN (40 kDa) administered at a dose of 135 μg weekly for 48 mo was efficacious but not perfectly tolerable in dialysis patients with chronic hepatitis C.

Research frontiers

The present study was carried out in South-east Anatolien/Diyarbakir, Turkey, in hemodialysis patients with chronic hepatitis C.

Related publications

Yu JW, Wang GQ, Sun LJ, Li XG, Li SC. Predictive value of rapid virological response and early virological response on sustained virological response in hepatitis C virus (HCV) patients treated with pegylated interferon α-2a and ribavirin. *J Gastroenterol Hepatol*. 2007 Jun; 22(6): 832-836. Sporea I, Popescu A, Sirli R, Golea O, Totolici C, Danila M, Vernic C. Pegylated-interferon α 2a treatment for chronic hepatitis C in patients on chronic hemodialysis. *World J Gastroenterol*. 2006 Jul 14; 12(26): 4191-4194.

Innovations and breakthroughs

Many studies published that PEG-IFN treatment is efficacious and tolerable in hemodialysis patients with chronic hepatitis C. In our study we found similar efficacy, but this treatment was not perfectly tolerable in dialysis patients with HCV infection.

Peer review

Ayaz *et al* investigated the effects of PEG-IFN for treatment of HCV infection in hemodialysis patients. Eleven of 17 patients had a SVR at wk 24 after end-of-treatment. In 5 patients treatment had to be stopped because of side effects. The currently available data on this topic are scarce and therefore this is an important study which should be published.

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RAPID COMMUNICATION

Role of echo Doppler ultrasonography in the evaluation of postprandial hyperemia in cirrhotic patients

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Abstract

AIM: To assess the role of echo-Doppler ultrasonography in postprandial hyperemia in cirrhotic patients by comparing the results with the hepatic vein catheterization technique.

METHODS: Patients with cirrhosis, admitted to the portal hemodynamic laboratory were included into the study. After an overnight fast, echo-Doppler ultrasonography (basal and 30 min after a standard meal) and hemodynamic studies by hepatic vein catheterization (basal, 15 min and 30 min after a standard meal) were performed. Ensure Plus (Abbot Laboratories, North Chicago, IL) was used as the standard liquid meal. Correlation analysis of the echo-Doppler and hepatic vein catheterization measurements were done for the basal and postprandial periods.

RESULTS: Eleven patients with cirrhosis (5 Child A, 4 Child B, 2 Child C) were enrolled into the study. After the standard meal, 8 of the 11 patients showed postprandial hyperemia with increase in portal blood flow, portal blood velocity and hepatic venous pressure gradient. Hepatic venous pressure gradient in the postprandial period correlated positively with postprandial portal blood velocity (r = 0.8, P < 0.05) and correlated inversely with postprandial superior mesenteric artery pulsatility index (r = -1, P < 0.01).

CONCLUSION: Postprandial hyperemia can be efficiently measured by echo-Doppler ultrasonography and the results are comparable to those obtained with the hemodynamic studies.

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Key words: Postprandial hyperemia; Echo-Doppler; Hemodynamic study; Cirrhosis; Portal hypertension; Portal pressure

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INTRODUCTION

It is well known that portal pressure increases during the postprandial period. It is caused by an augmentation of blood flow into the splanchnic area, a phenomenon known as "postprandial hyperemia"[1,2]. In portal hypertensive patients, postprandial hyperemia may increase the variceal wall tension and trigger acute variceal bleeding. Therefore, an assessment of postprandial hyperemia (PPH) in cirrhotic patients may provide a better understanding of the pathophysiology of variceal bleeding, and consequently impact our treatment strategy.

Hepatic venous pressure gradient measurement by hepatic vein catheterization is the gold standard technique for the assessment of portal pressure changes in postprandial period in patients with cirrhosis[3]. However, this procedure is costly, is uncomfortable for the patients and is not available in every hepatology unit. By contrast, echo-Doppler ultrasonography (USG) is inexpensive, easy for the patients and is available in most hospitals. Sabba *et al* studied the sensitivity of echo-Doppler USG in detecting hemodynamic changes caused by postprandial hyperemia in an observer-blinded study. They found that echo-Doppler USG was highly sensitive for the evaluation of postprandial hyperemia[4]. Additionally, several studies have been done to investigate the effects of different drugs on postprandial hyperemia by using echo-Doppler USG[5-8]. However, to the best of our knowledge, there is no study which compares the hepatic venous catheterization with echo-Doppler USG in the assessment of PPH in patients

with cirrhosis. Therefore, the aim of present study was to investigate the sensitivity of echo-Doppler USG in the assessment of PPH by comparing results with the gold standard test, hepatic vein catheterization.

MATERIALS AND METHODS

Patients

All cirrhotic patients who were admitted to the portal hemodynamic laboratory of Marmara University School of Medicine, Department of Gastroenterology from November 2003 to March 2004, for the hemodynamic measurements before initiation of primary or secondary variceal bleeding prophylaxis were evaluated for the enrollment into the study. The exclusion criteria were age less than 18 or greater than 80 years, presence of hepatocellular carcinoma, coronary heart disease, hypertension, diabetes mellitus, portal vein thrombosis, refractory ascites, chronic lung disease, hepatic encephalopathy, hepatorenal syndrome and refusal to participate in the study.

The study was approved by the ethical committee of Marmara University School of Medicine and all patients gave written informed consent prior to participating in the study.

Study design

Diuretics and beta-blocker drugs were stopped 3 d before the study. After an overnight fast, baseline echo-Doppler (ATL, Ultramark 8 echo-Doppler duplex system) study was performed and the systolic (SBP), diastolic blood pressure (DBP) and heart rate (HR) were obtained. After these procedures, the patients were prepared for hepatic vein catheterization to obtain baseline hemodynamic measurements. Right hepatic vein catheterization was performed percutaneously through the jugular vein and the pressures were recorded in both the wedged and free position, using a balloon catheter (Medi Tech, Cooper Scientific Corp, Watertown, MA). The wedged position was controlled by the absence of reflux after the injection of 2 mL of contrast medium. Pressures were measured by a strain gauge transducer previously calibrated, and the findings were recorded on paper. Each pressure reading was recorded twice and the mean of the two values was used for the final result.

After the baseline measurements were obtained, a standard mixed liquid meal was given which consisted of 237 mL, of balanced dietary supplement (Ensure Plus, Abbot Laboratories, North Chicago, IL), containing 355 kcal as 14.7 g protein, 53.3 g carbohydrate and 32 g lipid. The meal was consumed over a period of 10 min. The mixed liquid meal was chosen because of ease of consumption, and standard formula and calorie intake. Hemodynamic measurements were repeated at 15, 30 and 60 min after the meal. At 30th min after the meal, echo-Doppler USG measurements were repeated simultaneously with the hemodynamic study. Because of technical difficulties (transport of the patients to the Doppler room), echo-Doppler USG could not be performed at the 15th and 60th min of postprandial period.

Parameters

Mean arterial pressure (MAP) = (systolic + diastolic × 2)/3.

Hemodynamic studies

Hepatic venous pressure gradient (HVPG) = Wedge hepatic venous pressure (WHVP) - Free hepatic venous pressure (FHVP).

Echo-Doppler USG

Portal Blood Flow (PBF) = Portal vein cross-sectional area (A) mm^2 × Portal blood velocity (PBV) cm/s.

An area was obtained from the cross-section of the vessel visualized by B-mode after defining the major and minor diameters of the vessel. Velocity measurements were performed in quiet suspended inspiration and averaged over a few seconds. These measurements were obtained from the longitudinal section of the vessel by positioning the sample volume cursor over the vessel at a known angle of insonation, defined as an angle between the Doppler beam and the long axis of the vessel. The Doppler sample volume probe was positioned over the middle of the vessel and was manipulated to cover about 90% of the vessel section in order to detect the maximum Doppler frequency shift, related to the maximum velocity (4).

According to the Doppler equation:

$$\Delta f = \frac{2 \text{ foV max cos Q}}{c}$$

c: speed of the sound in the tissue

Q: angle between the ultrasound beam and the director of the blood flow (angle of insonation)

$$Vm = 0.57 \times Vmax$$

Additionally, pulsatility index (PI) for the superior mesenteric artery (SMA) was measured according to the formula:

PI = Vmax - Vmin/Vmean

To decrease the inter-observer variability, all measurements were repeated 3 times by 2 different investigators and the mean value was used for statistical analysis.

Statistical analysis

Baseline results for the measurements are given as mean ± SE of absolute values. The values over time (Basal, 15 and 30 min after the test meal) were compared by ANOVA and the Student's *t* test. Spearman correlation tests were done between the measurements of echo-Doppler USG and hemodynamic studies.

RESULTS

One hundred patients attending the hepatology outpatient clinic of the Marmara University Hospital were screened. However, only 11 patients were included in the study; the remainder were excluded due to factors such as clinical status, high Child-Pugh score, hematologic parameters, patients not providing informed consent and other exclusion criteria. The characteristics of the 11 patients are shown in Table 1. Eight out of the 11 patients showed postprandial hyperemia as judged by

Table 1 Demographic characteristics of the study patients

Age	48 ± 11.2
Male/Female	8/3
Alcoholic etiology (yes/no)	2/9
Child-Pugh Score	7.6 ± 2.3
Child-Pugh Class (A/B/C)	5/4/2
History of variceal bleeding	
Yes	6
No	5
History of encephalopathy	
Yes	4
No	7
History of ascites	
Yes	6
No	5
History of SBP	
Yes	2
No	9
ALT (IU/L)	52.27 ± 24.2
Total protein (g/L)	6.97 ± 0.9
Albumin (g/d)	3.52 ± 0.5
PT (s)	17.3 ± 2.9
Total bilirubin (mg/dL)	1.81 ± 0.5

ALT: Alanine aminotransferase; PT: Prothrombin time.

Table 2 Hemodynamic and echo-Doppler measurements of the study patients

	Baseline	Postprandial (15 min)	Postprandial (30 min)	*P*
Hemodynamic measurements				
FHVP (mmHg)	14.2 ±3.6	15.2 ± 4.1	15.9 ± 5.9	NS
WHVP (mmHg)	30.5 ± 7.6	38.7 ± 11.8[b]	37.1 ± 10.4	< 0.05
HVPG (mmHg)	16.5 ± 6.7	23.7 ± 11.1[d]	21.2 ± 9.5	< 0.05[a]
Echo-Doppler measurements				
PBF (L/min)	1.5 ± 0.9		2.4 ± 1.1	< 0.05
PBV (cm/s)	23.5 ± 11.6		32.9 ± 12.3	< 0.01
SMA-V (cm/s)	120.2 ± 23.3		158.1 ± 59.9	< 0.05
SMA-PI	2.8 ± 0.5		1.7 ± 0.2	< 0.05
Systemic measurements				
MAP (mmHg)	85 ± 12.9		80 ± 9.6	< 0.05
Pulse Rate (min)	89.70 ± 21.2		85.3 ±13.3	< 0.05

[b]$P < 0.01$ postprandial 15 min *vs* basal hepatic venous pressure gradient (HVPG); [a]ANOVA statistics of basal, 15 min and 30 min measurements of hepatic vein catheterization; [d]$P < 0.01$ postprandial 15 min HVPG *vs* basal HVPG. FHVP: Free hepatic venous pressure; WHVP: Wedge hepatic venous pressure; PBF: Portal Blood Flow; PBV: Portal blood velocity; SMA: Superior Mesenteric Artery; PI: Pulsatility index; V: Velocity; MAP: Mean arterial pressure.

both echo-Doppler and hemodynamic studies, shown in Table 2. The maximum postprandial hyperemia was obtained at the 15 min. measurement by hepatic vein catheterization. Three patients did not show any increase in the hemodynamic or echo-Doppler parameters during the postprandial period (data not shown).

When compared with the baseline parameters, there was a statistically significant difference in WHVP, HVPG, PBF, PBV, SMA-V, SAMA-PI, MAP and pulse rate at the 15th and 30th min of the postprandial period, with the portal vein velocity showing the highest increase ($P < 0.01$) (Table 2).

Spearman correlation tests were performed in order to evaluate the correlation between the echo-Doppler and hemodynamic parameters. Postprandial PBV showed a positive correlation with post-prandial HVPG values ($r = 0.8$, $P < 0.05$) (Figure 1), whereas SMA-PI showed an inverse correlation with the HVPG ($r = -1$, $P < 0.01$) (Figure 2). The percent increase in the echo-Doppler and hemodynamic measurements did not show any correlation between the echo-Doppler and hemodynamic tests.

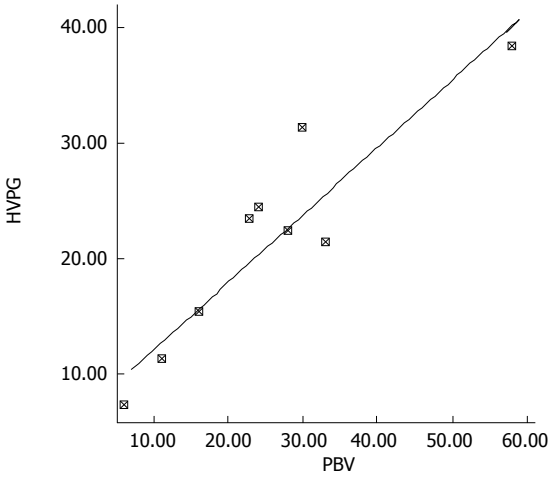


Figure 1 Positive correlation between the portal blood velocity (PBV) and hepatic venous pressure gradient (HVPG) ($r = 0.8$, $P < 0.05$).

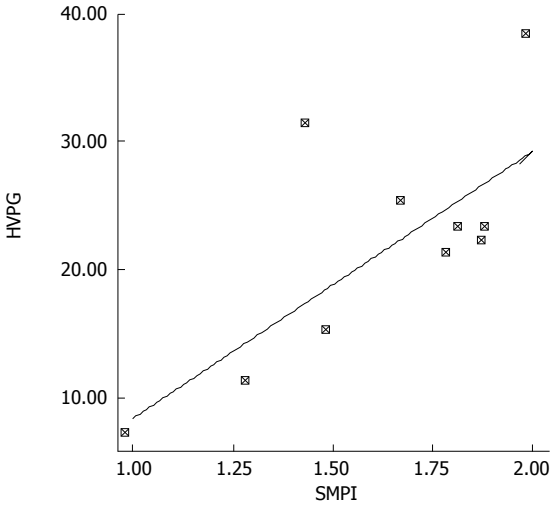


Figure 2 Inverse correlation between the superior mesenteric artery pulsatility index (SMA-PI) and hepatic venous pressure gradient (HVPG) ($r = -1$, $P < 0.01$).

DISCUSSION

The development of a non-invasive test for monitoring portal hemodynamics has been the subject of debate ever since the importance of portal pressure in cirrhotic patients was established. The increase in portal pressure in the postprandial period may initiate variceal bleeding in cirrhotic patients[9]. Therefore, it is important to identify patients who experience increased postprandial hyperemic response, and treat these patients appropriately. Echo-Doppler ultrasonography is a non-invasive test which is widely used to determine portal blood flow[4,7,8,10] In an observer-blinded study, it was shown that echo-

Doppler USG is highly sensitive in detecting postprandial hemodynamic changes[4]. Since hepatic venous pressure gradient measurement is the gold standard for the evaluation of portal hemodynamic changes, the aim of the present study was to compare the results obtained with this technique with echo-Doppler ultrasonography.

We observed that patients who showed postprandial increase in HVPG also demonstrated an increase in the parameters by echo-Doppler ultrasonography. Initially, we did not find any correlation between the basal measurements obtained by echo-Doppler and hemodynamic studies. However, postprandial portal blood flow increased significantly in patients who showed significant postprandial HVPG increase. Moreover, correlation analysis showed a strong correlation between postprandial PBV and hepatic venous pressure gradient (Figure 1). The SMA index showed a negative correlation with the HVPG (Figure 2). However, we did not find any correlation between portal blood flow and hemodynamic measurements. Portal blood velocity and SMA-PI are both velocity measurements which were obtained by direct measurements. However, measurement of portal blood flow requires assessment of cross sectional portal vein area which depends on the position of the probe as well as that of the patient, which may result in marked variability of portal blood flow readings. It is difficult to explain the lack of correlation in the basal measurements obtained by echo-Doppler and hemodynamic studies, although there was a good correlation during the postprandial period. This may be due to individual differences in the basal parameters obtained by echo-Doppler studies, which is why the echo-Doppler studies cannot replace the gold standard hemodynamic studies in the primary and secondary prophylaxis of variceal bleeding.

Hepatic venous pressure gradient measurement is the gold standard for the evaluation of portal hemodynamics. To the best of our knowledge, the present study is the only one which has compared hepatic vein catheter and echo-Doppler measurements. Assessment of patients with significant increase in postprandial portal pressure allows the use of several treatment options. It was recently observed that administration of low dose isosorbide mononitrate attenuates postprandial hyperemia[10]. By contrast, propranolol is ineffective in blunting postprandial hyperemia but has some effect in reducing postprandial portal blood flow[11]. Somatostatin analogs have a favorable effect in reducing postprandial hyperemia in cirrhotic patients[12-14]. Most of these studies are performed by echo-Doppler measurement[8,12,13].

Another unexpected finding in the present study was the absence of postprandial hyperemia in 3 patients (data not shown). These 3 patients showed more collateral vessels in the splanchnic area on echo-Doppler studies, compared to the other patients. In a recent study, it was shown that the extent of collateral circulation influences postprandial increase in portal pressure in patients with cirrhosis[15]. Other factors such as circadian rhythm, posture, age and gender did not have any effect in patients who failed to show a significant increase in postprandial pressure[16-20]. We believe that an increase in collateral circulation prevents postprandial rise in portal pressure despite increased blood flow in the splanchnic area.

In conclusion, if properly performed, echo-Doppler ultrasonography is a good test to measure postprandial portal hemodynamics. Future studies are needed to assess the usefulness of echo-Doppler measurements in primary and secondary prophylaxis of variceal bleeding.

COMMENTS

Background

Echo-Doppler USG is widely used for evaluation of the mechanisms of postprandial hyperemia in healthy people and cirrhotic patients, although the findings have not been validated.

Research frontiers

This is the first study which investigated the validity of echo-Doppler USG in postprandial hyperemia by comparing the results with the gold standard test, hepatic vein catheterization.

Innovations and breakthroughs

Postprandial pressure and portal blood flow were evaluated in eleven patients enrolled from a cohort of cirrhotic patients, by hepatic vein catheterization and echo-Doppler USG at 15 min and 30 min after a standard liquid meal. Although, basal parameters did not show any correlation, hepatic venous pressure gradient and portal blood velocity showed a positive corelation in postprandial measurements.

Applications

Although there were some limitations in the present study, it can be stated that the evaluation of postprandial hemodynamic alterations can be performed safely by echo-Doppler USG, and the measurements obtained by echo-Doppler study reflect accurately the postprandial parameters in cirrhotic patients.

Peer review

This is an experimental work conducted in humans, comparing hepatic vein catheterization with echodoppler measurement of post prandial hyperemia in cirrhotics. The authors conclude that the increased portal pressure observed in the post prandial period can be reliably measured by echo-doppler. The work is novel and may be clinically relevant.

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RAPID COMMUNICATION

Is obesity associated with gastropharyngeal reflux disease?

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Abstract

AIM: To examine the association between obesity and gastropharyngeal reflux disease (GPRD) as well as gastroesophageal reflux disease (GERD)

METHODS: We conducted a cross-sectional study of consecutive patients undergoing ambulatory 24-h dual-probe pH monitoring from July 2003 to December 2006. The association between body mass index (BMI) and parameters about gastroesophageal or gastropharyngeal reflux was examined in univariate and multivariate analyses.

RESULTS: A total of 769 patients (307 men and 462 women; mean age 50.7 years) were finally enrolled. Most variables showing gastroesophageal reflux was higher in the obese patients than the patients with normal BMI. There was no difference in all the variables showing gastropharyngeal reflux according to the BMI. After adjustment for age, sex, alcohol intake and smoking, obese patients demonstrated an about 2-fold increase in risk of GERD compared with patients with normal BMI (OR, 1.9; 95 CI, 1.3-2.9), but overweight patients did not demonstrate increased risk of GERD (OR, 1.2; 95 CI, 0.8-1.7). Both obese patients and overweight patients did not demonstrated increased risk of GPRD compared with patients with normal BMI (OR, 1.1; 95 CI, 0.8-1.7; and OR, 0.9; 95 CI, 0.6-1.3, respectively).

CONCLUSION: Obesity is not associated with GPRD reflux while it is associated with GERD.

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Key words: Obesity; Body mass index; Gastroesophageal reflux; Gastropharyngeal reflux

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INTRODUCTION

The worldwide prevalence of being overweight and obesity has been increasing at an alarming rate over the last decade, indiscriminately affecting populations of both higher and lower middle income countries[1]. The rise in obesity coincides with rising prevalence of gastroesophageal reflux disease[2,3], and gastroesophageal reflux disease is a common disorder that has been linked to obesity.

Obesity is a postulated risk factor for gastroesophageal reflux disease, although individual studies have conflicting results[4-8]. Some studies suggest that an increased body mass index (BMI) is associated with increased esophageal acid exposure[9] and with an increased risk of hospitalization for esophagitis[10]. In contrast, other studies, including one of the largest population-based studies to date, have found no association between BMI and gastroesophageal reflux disease[11-13]. Potential explanations for the disparate results include a true lack of an association between BMI and gastroesophageal reflux disease, differences in definitions or methodology, dissimilar study populations, or a lack of power to detect an effect in some studies. Additionally, many studies assessing the relationship between gastroesophageal reflux disease and obesity are symptom-based and lack objective tests to confirm this association.

Gastropharyngeal reflux, also called laryngopharyngeal reflux, is a term used to describe esophageal acid reflux into the laryngeal and pharyngeal areas. It causes extraesophageal manifestations (e.g., chronic cough, hoarseness, asthma, globus sensation, chronic sinusitis, or other pulmonary or otorhinolaryngologic diseases). Currently, the best way to demonstrate gastropharyngeal reflux is ambulatory 24-h dual probe pH monitoring[14]. Up to present, there are few reports on the association between BMI and gastropharyngeal reflux disease[15].

Therefore, we conducted this cross-sectional study to

examine the association of BMI and gastropharyngeal reflux disease as well as gastroesophageal reflux disease by using the ambulatory 24-h dual probe pH monitoring.

MATERIALS AND METHODS

Study design

We conducted a cross-sectional study of consecutive patients who underwent ambulatory 24-h dual-probe pH monitoring from July 2003 to December 2006 at the motility laboratory in Pusan National University Hospital (Busan, Korea). The indications for ambulatory 24-h dual-probe pH monitoring were globus sensation (sensation of a lump, something sticking in the throat), hoarseness, chronic cough, halitosis, throat clearing and laryngeal pathology such as vocal polyp. We did not enroll patients who had history of gastric surgery, were diagnosed as scleroderma or achalasia, or were on anti-reflux medications at the time of the study.

This study was reviewed and approved by the Institutional Review Board at Pusan National University Hospital.

Evaluation of body mass index

Body mass index (BMI) was calculated as body weight (kg) divided by the square of standing height (m). The BMI was categorized into 3 levels according to the WHO for the Western Pacific region[16]: normal weight-BMI < 23 kg/m^2, overweight-BMI $\geqslant$ 23 kg/m^2 and $\leqslant$ 25 kg/m^2, obese-BMI > 25 kg/m^2.

Esophageal manometry

All antisecretory and prokinetic medications were discontinued at least 7 d before testing. Esophageal manometry was performed, after an overnight fast, using an eight-lumen catheter (Synetics Medical Co., Stockholm, Sweden) with side holes 3, 4, 5, 6, 8, 13, 18, and 23 cm from the catheter tip and a water-perfused, low-compliance perfusion system (Synetics Medical Co., Stockholm, Sweden), according to a standard protocol. Briefly, the manometry protocol included the following: first, a station pull-through was performed through the lower esophageal sphincter (LES) to determine the end-expiratory resting pressure, LES length, and location relative to the nares. Then the catheter was positioned with the most-distal side-hole 2 cm below the upper margin of the LES. Ten 5-mL water swallows were given to evaluate peristalsis; only esophageal body contractions measured at 3, 8, and 13 cm above the LES were recorded for data analysis. Then the catheter was pulled through the upper esophageal sphincter (UES) in the same manner (station pull-through) to determine the resting UES pressure, length, and location relative to the nares. Esophageal manometric abnormalities were classified as achalasia, diffuse esophageal spasm, nutcracker esophagus, isolated hypertensive LES, ineffective esophageal motility, or nonspecific esophageal motility disorder[17].

Ambulatory 24-h dual-probe pH monitoring

Ambulatory 24-h dual-probe pH monitoring was performed immediately after esophageal manometry, with using a single-use monocrystalline antimony dual-site pH probe (Zinetics 24, Medtronic Inc., Minneapolis, USA) with electrodes placed at the tip and 15 cm proximal to the tip. A cutaneous reference electrode placed on the upper chest was also used. All the electrodes were calibrated in buffer solutions of pH 7 initially and then pH 1. The pH catheter was introduced transnasally into the stomach and withdrawn back into the esophagus until the electrodes were 5 cm above the proximal margin of the LES. The subjects were encouraged to eat regular meals with restriction for intake of drink or food with a pH below 4. All the subjects recorded their meal times (start and end), body position (supine and upright), and any symptoms in a diary. The data was collected using a portable data logger (Digitrapper Mark Ⅲ, Synetics Medical Co., Stockholm, Sweden) with a sampling rate of 4 s, and was transferred to a computer for analysis using "Polygram for Windows release" 2.04 (Synetics Medical Co., Stockholm, Sweden). For both sites, a decrease in pH below 4, which was not induced by eating or drinking, was considered the beginning of a reflux episode, and the following rise to pH above 4 was considered the end of such an episode. To be accepted as a gastropharyngeal reflux event, the decrease at the proximal probe had to be abrupt and simultaneous with the decrease in the esophagus, or to be preceded by a decrease in pH of a similar or larger magnitude in the distal probe. Thus, acid episodes induced by oral intake, aero-digestive tract residue and secretions, proximal probe movement, or loss of mucosal contact in which the proximal pH decline may precede the esophageal pH drop were not included as gastropharyngeal reflux episodes.

The variables assessed for gastroesophageal reflux in the distal probe were the total percentage of time the pH was < 4, the percentage of time the pH was < 4 in the supine and upright positions, the number of episodes the pH was < 4, the number of episodes the pH was < 4 for $\geqslant$ 5 min, the duration of the longest episode the pH was < 4 and the DeMeester composite score[18].

The variables assessed for gastropharyngeal reflux in the proximal probe were the total percentage of time the pH was < 4, the percentage of time the pH was < 4 in the supine and upright positions, and the number of episodes the pH was < 4.

For the diagnosis of gastroesophageal reflux disease (GERD) in the distal probe, two different aspects were analyzed[19,20]: (1) total reflux time: the total proportion of the recorded time with pH < 4; a value of > 4 was considered abnormal; (2) number of reflux episodes: the total number of pH episodes with pH < 4 during the recording; a value of > 35 episodes was considered abnormal.

For the diagnosis of gastropharyngeal reflux disease (GPRD) in the proximal probe, we considered more than 0.1 for the total, 0.2 for the upright, and 0 for the supine time of pH < 4 to be pathological. For the number of reflux episodes, more than 4 reflux episodes were considered pathological[21,22].

Assessment by endoscopy

The presence or absence of reflux esophagitis,

Table 1 Patient profiles and the endoscopic findings according to the body mass index

	Body mass index (kg/m^2)			*P* value
	< 23 (*n* = 344)	23-25 (*n* = 231)	>25 (*n* = 194)	
Age (yr, mean ± SEM)	48.4 ± 0.7	51.5 ± 0.7	53.8 ± 0.8	< 0.001
Gender (%)				0.077
Men	123 (35.8)	104 (45.0)	80 (41.2)	
Women	221 (64.2)	127 (55.0)	114 (58.8)	
Alcohol intake	66 (19.2)	50 (21.6)	55 (28.4)	0.048
Smoking	42 (12.2)	31 (13.4)	33 (17.0)	0.295
Indication for pH monitoring (%)				0.210
Globus	156 (45.3)	100 (43.3)	86 (44.3)	
Hoarseness	76 (22.1)	60 (26.0)	54 (27.8)	
Coughing	54 (15.7)	46 (19.9)	26 (16.4)	
Others[1]	58 (16.9)	25 (10.8)	28 (14.4)	
Endoscopic findings (%)				
Reflux esophagitis[2]	32/291 (11.0)	19/204 (9.3)	32/166 (19.3)	0.009
Hiatal Hernia	15/291 (5.2)	19/204 (9.3)	12/166 (7.2)	0.199
Endoscopically suspected esophageal metaplasia	18/291 (6.2)	18/204 (8.8)	16/166 (9.6)	0.348

[1]Other indications were halitosis, throat clearing and laryngeal pathology such as vocal polyp; [2]Los Angeles classification grade.

endoscopically suspected esophageal metaplasia and hiatal hernia were determined by two endoscopists (G.H. Kim, G.A. Song), who were blind to the information of the ambulatory 24-h pH monitoring.

Reflux esophagitis

If esophagitis was present, it was graded according to the Los Angeles classification[23].

Hiatal hernia

Hiatal hernia was defined as a circular extension of the gastric mucosa above the diaphragmatic hiatus greater than 2 cm in the axial length.

Endoscopically suspected esophageal metaplasia (ESEM)

The presence or absence of ESEM[24] was examined in the lower portion of the esophagus, including the esophagogastric junction, during inflation of the esophagus before inserting the endoscope into the stomach. The esophagogastric junction was defined as the oral side end of the fold, which exists continuously from the gastric lumen[25], as well as the end of the anal side of the fine longitudinal vessel, because the veins in the lower part of the esophagus were distributed uniformly, running parallel and longitudinally in the lamina propria[26,27]. The squamo-columnar junction was defined by a clear change in the color of the mucosa. ESEM was defined as the area between the squamo-columnar junction and the esophagogastric junction.

Statistical analysis

Data were expressed as mean ± SE unless otherwise noted. The differences in gender, alcohol intake, smoking, indication for pH monitoring, reflux esophagitis, hiatal hernia, ESEM, manometric diagnosis, GERD and GPRD according to the BMI were assessed using the χ^2 test. The one-way ANOVA was used to assess statistical significance for age, parameters of esophageal manometry and parameters of ambulatory pH monitoring according to the BMI and post-hoc analysis was performed using the Tukey's HSD. Multiple logistic regression analyses were used to examine association of the two primary outcomes (GERD, GPRD) with the main predictor variable, BMI. GERD and GPRD was adjusted for age, sex, alcohol intake and smoking. For all models, the number of covariates examined was determined by the number of outcome events with 10 events required for one covariate[28]. Patients with normal BMI constituted the reference group in comparisons between BMI levels. Odds ratios (ORs) and their 95 confidence intervals (CIs) were used to assess the association between BMI and GERD or GPRD, defined by ambulatory pH monitoring. A $P < 0.05$ was considered statistically significant. Statistical calculations were performed using the SPSS version 12.0 for Windows software (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 769 patients were enrolled in the study: 307 men and 462 women, and their mean age was 50.7 years. Of them, 661 patients underwent the upper endoscopy. Obese patients were more likely to be older and alcohol drinker. There was no difference in the hiatal hernia and ESEM according to the BMI. Reflux esophagitis was higher in the obese patients than the patients with normal BMI and the overweight patients ($P < 0.05$) (Table 1).

There was no difference in the proximal esophageal amplitude, LES pressure and LES length according to the BMI. But the distal esophageal amplitude was higher in the obese patients than the patients with normal BMI and the overweight patients ($P < 0.05$). There was no significant difference in esophageal motility abnormalities according to the BMI (Table 2).

There was no difference in all the variables showing gastropharyngeal reflux in the proximal probe according to the BMI. The total and upright time of pH below 4, the number of reflux episodes, and the DeMeester composite score was higher in the obese patients than the patients with normal BMI ($P < 0.05$) (Table 3).

Table 2 Results of esophageal manometry according to the body mass index (mean ± SE)

	Body mass index (kg/m^2)			*P* value
	< 23 (*n* = 344)	23-25 (*n* = 231)	> 25 (*n* = 194)	
Proximal esophageal amplitude (mmHg)	59.6 ± 1.7	60.1 ± 2.0	62.1 ± 2.4	0.650
Distal esophageal amplitude (mmHg)	75.1 ± 2.0	79.5 ± 2.9	90.2 ± 3.1[a]	< 0.001
LES pressure (mmHg)	20.9 ± 0.4	20.3 ± 0.5	20.6 ± 0.6	0.711
LES length (cm)	3.3 ± 0.0	3.3 ± 0.0	3.3 ± 0.0	0.636
Peristalsis (%)	96.5 ± 0.6	95.2 ± 0.8	96.1 ± 0.7	0.640
Manometric diagnosis				0.440
Normal	143	80	84	
Diffuse esophageal spasms	4	1	1	
Nutcracker esophagus	16	15	17	
Hypertensive LES	2	2	2	
Ineffective esophageal motility	103	74	52	
Nonspecific esophageal motility disorder	76	59	38	

Post hoc analysis using Tukey's HSD: [a]$P < 0.05$ *vs* BMI < 23 and BMI 23-25.

Table 3 Results of ambulatory 24-h dual probe pH monitoring according to the body mass index (mean ± SE)

	Body mass index (kg/m^2)			*P* value
	< 23 (*n* = 344)	23-25 (*n* = 231)	> 25 (*n* = 194)	
Proximal probe				
Time pH < 4 (total)	0.7 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.502
Time pH < 4 (upright)	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.809
Time pH < 4 (supine)	0.6 ± 0.3	0.2 ± 0.1	0.1 ± 0.0	0.245
No. of reflux episodes	11.1 ± 1.8	8.4 ± 1.0	9.5 ± 1.1	0.452
Distal probe				
Time pH < 4 (total)	2.7 ± 0.2	3.7 ± 0.5	3.8 ± 0.3[a]	0.038
Time pH < 4 (upright)	3.9 ± 0.3	5.8 ± 0.9	5.6 ± 0.5[a]	0.021
Time pH < 4 (supine)	1.4 ± 0.3	2.2 ± 0.6	2.1 ± 0.3	0.211
No. of reflux episodes	39.1 ± 2.3	46.1 ± 3.1	59.4 ± 4.9[a,b]	< 0.001
No. of reflux episodes ⩾ 5 min	1.6 ± 0.2	1.6 ± 0.2	2.0 ± 0.3	0.477
Longest reflux episode (min)	8.6 ± 1.3	11.3 ± 2.4	11.1 ± 0.9	0.385
DeMeester composite score	11.0 ± 0.9	14.5 ± 1.7	15.7 ± 1.2[a]	0.016

Post hoc analysis using Tukey's HSD: [a]$P < 0.05$ *vs* BMI < 23; [b]$P < 0.05$ *vs* BMI 23-25.

Table 4 Association of body mass index with gastroesophageal reflux disease and gastropharyngeal reflux disease, defined by ambulatory pH monitoring

	Body mass index (kg/m^2)			*P* value
	< 23 (*n* = 344)	23-25 (*n* = 231)	> 25 (*n* = 194)	
Gastroesophageal reflux disease (%)				0.001
Absent	203 (59.0)	121 (52.4)	82 (42.3)	
Present	141 (41.0)	110 (47.6)	112 (57.7)	
Gastropharyngeal reflux disease (%)				0.162
Absent	143 (41.6)	103 (44.6)	69 (35.6)	
Present	201 (58.4)	128 (55.4)	125 (64.4)	

The frequency of GERD defined by the ambulatory pH monitoring was higher in obese patients, not in overweight patients than in the patients with normal BMI. There was no significant difference in the frequency of GPRD defined by the ambulatory pH monitoring according to the BMI (Table 4).

After adjustment for age, sex, alcohol intake and smoking, obese patients demonstrated an about 2-fold increase in risk of GERD defined by the ambulatory pH monitoring compared with patients with normal BMI (OR, 1.9; 95 CI, 1.3-2.9), but overweight patients did not demonstrate increased risk of GERD (OR, 1.2; 95 CI, 0.8-1.7). Both obese patients and overweight patients did not demonstrated increased risk of GPRD defined by the ambulatory pH monitoring compared with patients with normal BMI (OR, 1.1; 95 CI, 0.8-1.7; and OR, 0.9; 95 CI, 0.6-1.3, respectively) (Table 5).

DISCUSSION

In present study, we evaluated GERD and GPRD by objective mean (ambulatory 24-h dual-probe pH monitoring) in a large group of 769 patients. Obese patients demonstrated an about 2-fold increase in risk

Table 5 Multivariable analysis: unadjusted and adjusted analyses for relationships of the body mass index with gastroesophageal reflux disease and gastropharyngeal reflux disease, defined by ambulatory pH monitoring

	Body mass index (kg/m^2)		
	< 23	23-25	> 25
Gastroesophageal reflux disease			
Unadjusted OR (95 CI)	1	1.3 (0.9-1.8)	2.0 (1.4-2.8)
Adjusted OR (95 CI)[1]	1	1.2 (0.8-1.7)	1.9 (1.3-2.9)
Gastropharyngeal reflux disease			
Unadjusted OR (95 CI)	1	0.9 (0.6-1.2)	1.3 (0.9-1.9)
Adjusted OR (95 CI)[1]	1	0.9 (0.6-1.3)	1.1 (0.8-1.7)

[1]Adjusted for age, sex, alcohol intake and smoking.

of GERD defined by the ambulatory pH monitoring compared with patients with normal BMI, but overweight patients did not demonstrate increased risk of GERD. Also, most variables showing gastroesophageal reflux was higher in the obese patients than in the patients with normal BMI, but not in overweight patients than in the patients with normal BMI. These results were in accord with the previous report that all measures of esophageal acid exposure were observed only for obesity, but not for overweight when compared to normal BMI[29]. In addition, we analyzed the endoscopic findings according to the BMI. Reflux esophagitis was higher in the obese patients than in the patients with normal BMI and the overweight patients.

The mechanism by which obesity promotes GERD remains unclear. One potential mechanism is related to mechanical factors whereby an increase in abdominal fat leads to an increase in intragastric pressure[9,30,31], and increased frequency of transient lower esophageal sphincter relaxation[32]. Obese patients may have an increased risk for hiatal hernia, which has a role in initiating and promoting gastroesophageal reflux[33,34]. On the other reports[35,36], there was not statistically significant association between BMI and hiatal hernia, similar to our results.

We also assessed the degree of gastropharyngeal reflux according to the BMI. There was no difference in all the variables showing gastropharyngeal reflux in the proximal probe according to the BMI. Also, obese patients did not demonstrated increased risk of GPRD defined by the ambulatory pH monitoring compared with patients with normal BMI. These results were consistent with the only previous report[15] about the association of BMI and GPRD. In that report, the authors showed that obesity was not associated with the pharyngeal reflux events but had a significant association with esophageal reflux events. But they simply compared the mean pharyngeal and esophageal reflux numbers between 195 non-obese patients and 90 obese patients. Even though we demonstrated the similar result that GPRD was not associated with BMI, we included much more patents, categorized them into 3 levels (normal, overweight, obesity) and performed the multiple logistic regression analysis after adjustment for age, sex, alcohol intake and smoking. Also, our results were consistent with a prospective study[37] that obesity was not risk factors for the occurrence of extraesophageal disorders after multivariate analysis, although the presence of extraesophageal disorders was assessed by only a questionnaire.

The prevalence of esophageal motility abnormalities according to the BMI is not yet unknown. In present study, there was no significant difference in esophageal motility abnormalities according to the BMI. Hong *et al*[38] showed the increased distal esophageal amplitude in 33 of morbidly obese patients and they suggested that this might be due to the presence of a high intraabdominal-thoracic pressure gradient in morbidly obese patients. This would cause a functional outflow obstruction of the esophagus, creating a high-pressure zone within the esophagus. In response to this chronic high-pressure zone, the distal esophagus would have to produce high amplitude contractions for passage of oral contents into the stomach. Similarly, in present study, the distal esophageal amplitude was higher in the obese patients than in the patients with normal BMI and the overweight patients. There was no difference in the LES pressure and LES length according to the BMI, which is consistent with previous reports[9,39].

Much more controversy exits about the location of the proximal probe. The recording of the pH in the hypopharynx is technically difficult. Acid exposure in the hypopharynx can easily be missed because of the relatively large space within the hypopharynx[21]. On the contrary, placement of the proximal probe in or below the upper esophageal sphincter allows a more permanent contact with the mucosa during the 24-h period resulting in fewer artifacts[21,22]. We used the dual-site pH probe with electrodes placed at the tip and 15 cm proximal to the tip, and we could not choose the exact location of proximal probe. But in most cases (72.7, 559/769), the proximal probe was located in the upper esophageal sphincter. So, for the diagnosis of GPRD, we used the criteria proposed by Smit *et al*[21,22].

There were some limitations in this study. First, ambulatory pH monitoring can be subjected to measurement errors related to placement of the probe and instrument calibration. However, all procedures were conducted using a similar technique with single-use pH probe. Another limitation is the absence of systematic collection of GERD symptoms. Because our main focus was to examine objective evidence of GERD (i.e., ambulatory pH monitoring) according to the BMI, we did not administer structure questionnaire. Nevertheless, this study had several advantages including the prospective design including the measurement of weight, height, alcohol intake and smoking, the consecutive enrollment to reduce the impact of selection bias and the large sample size. In addition, numerous patients (661/769) underwent the upper endoscopy and we could analyze the endoscopic findings according to the BMI.

Why is obesity not associated with GPRD despite of the association with GERD? First, acid refluxed into esophagus is usually cleared by gravity and peristaltic contractions. In obese patients, the esophageal peristaltic contraction is not impaired compared with patients with normal BMI. On the contrary, the distal esophageal amplitude is increased. This fact would play some role in preventing the refluxed acid extending to the upper level. Second, the amount of acid refluxed into esophagus is

increased in obese patients, so it is easily assumed that the amount of acid refluxed into the upper level would be increased by a secondary phenomenon. The refluxed acid is neutralized by esophageal submucosal secretions and swallowed salivary secretions, so it becomes non-acid reflux material. Therefore, even though this non-acid refluxate would reach to the upper level, the proximal pH probe cannot detect it. To solve this problem, a prospective study using a combined multichannel intraluminal impedance and pH measurement which is able to detect both acid and non-acid reflux, as well as the proximal extent of the refluxate, will be needed.

In summary, this is the largest study to evaluate GERD and GRPD simultaneously according to the BMI by using the ambulatory 24-h dual-probe pH monitoring. Obesity is associated with GERD but is not associated with GPRD. Further studies using a combined multichannel intraluminal impedance and pH measurement will be needed.

COMMENTS

Background

Obesity is a postulated risk factor for gastroesophageal reflux disease, although individual studies have conflicting results. Some studies suggest that an increased body mass index (BMI) is associated with increased esophageal acid exposure and with an increased risk of hospitalization for esophagitis. In contrast, other studies have found no association between BMI and gastroesophageal reflux disease. Gastropharyngeal reflux is a term used to describe esophageal acid reflux into the laryngeal and pharyngeal areas. It causes extraesophageal manifestations such as chronic cough, hoarseness, asthma, globus sensation, chronic sinusitis, or other otorhinolaryngologic diseases. This study was to examine the association of BMI and gastropharyngeal reflux disease as well as gastroesophageal reflux disease.

Research frontiers

The research front in this area is focused on the association of BMI and gastropharyngeal reflux disease as well as gastroesophageal reflux disease. There have been many debates about the association of obesity with gastroesophageal reflux disease. Up to present, there are few reports on the association between BMI and gastropharyngeal reflux disease. This study has examined the association of BMI and gastropharyngeal reflux disease as well as gastroesophageal reflux disease by using the ambulatory 24-h dual-probe pH monitoring. Obesity was associated with increased risk of gastroesophageal reflux disease but was not associated with increased risk of gastropharyngeal reflux disease.

Innovations and breakthroughs

There are few reports on the association between BMI and gastropharyngeal reflux disease. Most previous studies about the association of obesity with gastroesophageal reflux disease are symptom-based and lack objective tests such as ambulatory 24-h pH monitoring. This study is the largest study to evaluate gastroesophageal reflux disease and gastropharyngeal reflux disease simultaneously according to the BMI by using the ambulatory 24-h dual-probe pH monitoring.

Applications

Obesity is associated with GERD but is not associated with GPRD. Further studies using a combined multichannel intraluminal impedance and pH measurement will be needed.

Terminology

Esophageal acid reflux into the laryngeal and pharyngeal areas causes extraesophageal manifestations such as chronic cough, hoarseness, asthma, globus sensation, chronic sinusitis, or other otorhinolaryngologic diseases. This condition is called as gastropharyngeal reflux disease.

Peer review

This manuscript describes a well-designed GPRD. The classification of obesity is much higher in the United States and Europe, so this may be an issue that warrants attention. Nonetheless, this is important research.

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RAPID COMMUNICATION

Referral for anorectal function evaluation is indicated in 65% and beneficial in 92% of patients

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Abstract

AIM: To determine the indicated referrals to a tertiary centre for patients with anorectal symptoms, the effect of the advised treatment and the discomfort of the tests.

METHODS: In a retrospective study, patients referred for anorectal function evaluation (AFE) between May 2004 and October 2006 were sent a questionnaire, as were the doctors who referred them. AFE consisted of anal manometry, rectal compliance measurement and anal endosonography. An indicated referral was defined as needing AFE to establish a diagnosis with clinical consequence (fecal incontinence without diarrhea, 3rd degree anal sphincter rupture, congenital anorectal disorder, inflammatory bowel disease with anorectal complaints and preoperative in patients for re-anastomosis or enterostoma, anal fissure, fistula or constipation). Anal ultrasound is always indicated in patients with fistula, anal manometry and rectal compliance when impaired continence reserve is suspected. The therapeutic effect was noted as improvement, no improvement but reassurance, and deterioration.

RESULTS: From the 216 patients referred, 167 (78%) returned the questionnaire. The referrals were indicated in 65%. Of these, 80% followed the proposed advice. Improvement was achieved in 35% and a reassurance in 57% of the patients, no difference existed between patient groups. On a VAS scale (1 to 10) symptoms improved from 4.0 to 7.2. Most patients reported no or little discomfort with AFE.

CONCLUSION: Referral for AFE was indicated in 65%. Beneficial effect was seen in 92%: 35% improved and 57% was reassured. Advice was followed in 80%. Better instruction about indication for AFE referral is warranted.

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INTRODUCTION

Anorectal function evaluation (AFE) consists of several tests. Institutions differ in their selection of tests[1]. At our tertiary centre, anal manometry, rectal compliance measurement and anorectal endosonography are performed as part of our standard procedure[2]. Defecography and colon transit time are performed on strict indications. Neurophysiological tests of the pelvic floor are performed only for research purposes. Anal manometry establishes anal pressures while rectal compliance measures sensitivity and the volume of the rectum. Anal endosonography visualizes possible defects or atrophy of the anal sphincter complex. AFE is often requested in patients with anorectal symptoms including fecal incontinence, anal soiling, fistulas, anorectal tumours, anal pain, constipation *etc*. AFE is available in a limited number of hospitals, mainly academic centres and some large peripheral clinics.

A clinical referral (no research purposes) is indicated when disease can be demonstrated or excluded on the basic of AFE and when it has further therapeutical consequences. Which patients benefit most from anorectal function tests (by reduction of symptoms or reassurance) is unclear. Literature concerning this issue is scarce. Most studies that mention anorectal function tests in relation to anorectal pathology limit themselves to pre- and post-treatment results. Therefore, it often remains unclear whether AFE leads to relevant findings or subsequent change of therapy[1-10]. A large multi-centre Dutch study referred to the value of AFE for outcome of physiotherapy in patients with fecal incontinence[1]. One conclusion was that AFE had no predictive value for outcome of physiotherapy. Further, referral for AFE largely depended on availability of these tests in the referring hospital.

The aim of this study was to determine the indicated referrals to our tertiary center for patients with anorectal symptoms, the effect of the advice on their complaints and the perceived discomfort for the patients during AFE.

MATERIALS AND METHODS

Patients

All patients who were first clinical referrals for AFE between May 2004 and October 2006 were selected from our database. The database contained the complete medical history and extensive data of anorectal symptoms and anorectal test results. Deceased patients were excluded. All patients were sent a questionnaire. Additional data about follow-up in the outpatient clinic, hospital admittance, diagnostic and therapeutic procedures performed in our hospital could be retrieved from the (electronic) patient hospital files.

The Medical Ethical Commission of the VU University Medical Centre granted permission.

The referring doctors

The doctors who referred patients in the study period were also sent a questionnaire.

Anorectal function evaluation (AFE)

This consisted of anal manometry, rectal compliance measurement and anorectal endosonography according to our methods previously described elsewhere[11].

Indicated referral

A referral is indicated when disease can be demonstrated or excluded on the basic of AFE and when it has further therapeutical consequences. These are patients with fecal incontinence without diarrhea, 3rd degree sphincter rupture with or without fecal incontinence, congenital disorders, patients with inflammatory bowel disease with anorectal complaints and preoperative in patients for re-anastomosis or enterostoma, anal fissure or constipation. In patients with fistula, an ultrasound is always indicated but anal manometry and rectal compliance measurement only on indication regarding fecal incontinence. Test results in all these patients influence management. In patients with constipation, AFE was considered indicated in suspected Hirschsprungs' disease and surgery. AFE was not considered indicated in patients with fissures treated conservatively, soiling (defined as anal discharge without overt fecal incontinence), anal pain and hemorrhoids, since results do not change management.

Questionnaires

The questionnaire for patients[2] contained questions about the actual received therapy and changes in their symptoms by the received treatment, stated in a Visual Analogue Score (VAS) (score 1-10, 1 = very bad, 10 = very good) and also stated as (1) improved, (2) no change but reassurance or acceptance of situation without further need for seeking other medical advice and (3) worse and/or no reassurance. Discomfort and pain during the examination was scored with VAS (score 1-10, 1 = very uncomfortable/painful, 10 = no discomfort/pain).

The questionnaire for the referring doctor[2] consisted of questions about implementing the advice (yes, no), the quality of the advice (good, neutral, poor) and the willingness to refer again (yes, no).

Treatment advice strategy

The patients with symptoms of fecal incontinence were divided into five diagnostic subtypes: incontinence due to a sphincter defect, neurogenic incontinence, combined incontinence (sphincter defect and neurogenic), incontinence due to small rectal capacity and incontinence due to diarrhea. Patients with incontinence due to diarrhea were advised to have the cause of their diarrhea treated by the referring doctor.

All patients with fecal incontinence were prescribed fibres and physiotherapy. When unsuccessful additional therapy was advised depending of the cause. Patients with a sphincter defect > 25% were offered a sphincter repair. In patients with a small rectal compliance an enterostoma was proposed (< 60 mL) or strongly recommended (between 60 and 100 mL)[12].

Patients with a known 3rd degree sphincter rupture and as a result fecal incontinence were advised as other patients with fecal incontinence and the strong advice for a cesarean section with a next childbirth. If they were not incontinent, depending on the size of the rupture, the possibility of a cesarean section for next childbirth was discussed.

Advising re-anastomosis or enterostoma depended on the total impression of the anorectal function measured with anal pressures, rectal compliance and sphincter defects or atrophy.

In patients with a fistula the extension of the fistula tract(s) with anal ultrasound determined the type of surgery in our hospital (fistulotomy in simple and curettage with mucosal advancement plasty in complicated fistulas).

Patients where AFE was not indicated also received an advice. In patients with constipation a fibre-enriched diet, additional fibres and laxatives were advised. When unsuccessful and not previously attempted, pelvic floor physiotherapy was advised. When constipation coexisted with complaints of prolapse, a defecography was advised. When surgery was considered (rectocele correction or colectomy) besides an AFE, also a colon transit time was performed. Patients with fissures were treated conservatively; when treatment failed they were referred to the surgeon and AFE was indicated. Hemorrhoids and mucosal prolapse were treated with rubber band ligation. A hemorrhoidectomy was advised only in refractory cases. Local causes of anal pain were treated according to their causes. When no local abnormalities were seen in patients with anal pain, fibres and referral to the anaesthesiologist was advised.

Statistical analysis

The results were described as mean with standard deviation. The χ^2 test for independence and for trend, the Kruskal-Wallis test and the Wilcoxon matched-pair test were used when appropriate (GraphPad InStat Software, San Diego, Ca, USA).

RESULTS

Response questionnaires

There were 216 first referrals for AFE, 181 (84%) females, mean age 51 years, (SD 15, range 15-82). Two patients had died. A total of 167 patients [137 females (82%), mean age 51 years, SD 15, range 16-82] returned an adequate (almost all questions answered) questionnaire (78%).

Indicated referrals

Table 1 shows the indicated referrals. Of the 167 referrals, 109 (65%) were indicated. The most frequent referral was fecal incontinence, from which 93% was indicated (7% had diarrhea).

Non-indicated referrals

Two of the 31 patients with constipation had signs of anismus during physical examination and anal manometry. Of the five patients with soiling, four had a mucosal prolapse and/or hemorrhoids. The fifth patient had an anal fissure on inspection, not previously found. In two patients with anal pain a fissure was found, one treated conservatively and one eventually much later with surgery. AFE revealed no abnormalities in all patients besides high rest pressure in the patients with fissures. AFE did not influence therapeutic advice.

Effect of treatment

Symptoms improved in 54 patients (35%). In 88 patients (57%) symptoms were unchanged but patients were reassured. Despite treatment, 12 patients (8%) deteriorated. The whole group improved one point on the VAS scale (5.1-6.1) ($P < 0.0001$), for those improved (35%) this was even 3.2 points (4.0-7.2). Both indicated and non indicated referred patients improved equally.

The causes of fecal incontinence were: sphincter defect (14), neurogenic (37), combined incontinence (10), incontinence due to diarrhea (5) and incontinence due to small rectal capacity (< 100 mL) (5). Within these groups, the largest improvement was seen in the combined incontinence group (1.8 point) ($P = 0.01$). Patients with a small rectal capacity had no improvement at all.

The actual therapies received by the patients according to the reason for referral are mentioned in Table 2. Some patients received several therapies. The most frequent advice was medication, mainly fibres.

Of all referred patients, only 17% were operated. No difference between effectiveness of conservative and surgical treatment could be observed on patient symptoms ($P = 0.09$).

AFE induced little stress, indicated by an average pain score of 7 (SD 2.7) and a discomfort score of 7.2 (SD 2.8). Two patients with fistulas experienced the examination as unpleasant and painful due to the hydrogen peroxide injection in their fistula tract during anal ultrasound. Thirty five females (26%) preferred to be examined by a female doctor while the remaining 102 (75%) had no preference. Twenty six males (93%) had no preference and the remaining two (7%) preferred a male and a female doctor, respectively ($P < 0.0001$). Dutch ethnic minorities did not influence these data.

Table 1 Indicated referrals in the main groups of patients

	All *n* (% of referrals)	Indicated *n* (% of that group)
Incontinence	71 (43)	66 (93)[1]
Constipation	31 (19)	6 (19)[2]
3rd sphincter rupture	21 (13)	21 (100)
Pain	9 (5)	0 (0)
Re-anastomosis/enterostoma	8 (5)	8 (100)
Soiling	5 (3)	0 (0)
IBD	4 (2)	4 (100)
Hemorrhoids	3 (2)	0 (0)
Anal atresia	2 (1)	2 (100)
Fistulas	2 (1)	2 (100)
Fissure	1 (1)	0 (0)
Other	9 (5)	0 (0)
Total	167 (100)	109 (65[3])

[1]Five patients with diarrhea not indicated; [2]Only patients suspected of Hirschsprung/surgery indicated, IBD-inflammatory bowel disease; [3]Percentage of all referrals.

Questionnaires referring doctors

Of the 214 questionnaires, 102 (48%) responses were obtained. The advice was nearly always implemented (96%). The quality of the advice was considered good in 76% and neutral in 24%. All doctors except one (98%) were willing to refer again.

Agreement between proposed and followed advice.

The proposed and followed therapies are shown in Table 3. Therapies could also be a combination of medication, physiotherapy or surgery. Dietary advice was always followed (100%), while surgical advice was generally followed (89%). Less accepted advice included medication (71%) and physiotherapy (73%) ($P = 0.005$, 99% CI).

DISCUSSION

The 78% response to the questionnaires of the patients was good. In our previous study we reported a similar result[2]. Only 65% of the referrals were indicated. In 35% the diagnosis could have been established by clinical examination or added nothing. This is a signal that more communication and education is warranted, especially in times with restrictions and limited resources. However, many of the referred patients suffered from chronic symptoms, bringing both patient and doctor to despair. The possibility of referring the patients to another centre may come as a welcome alternative. The symptoms of the whole group improved an average of one point from 5.1 to 6.1 on a ten-point scale. Actual improvement took place in 35% of the referred patients; they improved an average of 3.2 points. The moderate improvement might be explained by the fact that it concerned patients with chronic disorders, already treated conservatively for a long time. Success was not related to a specific symptom, diagnosis or treatment, only the five patients with fecal incontinence due to small rectal capacity did not improve. Deterioration in 8% of the patients was mainly due to the fluctuating course of the chronic complaints combined with their reluctance to follow the advice. In 80% of patients, advice was followed. Medication and

Table 2 Reason for referral and effect of treatment on patients

2004-2006		Treatment according to the patients						Symptoms change after treatment				
								VAS		Category		
Reason for referral	Patients	Treatment[1]	Diet *n* (%)	Medication *n* (%)	Surgery *n* (%)	Physiotherapy *n* (%)	Expectative *n* (%)	Before	After	Improved	Reassured	Worse
Incontinence[2]	71	100	8	32	12[3]	26	22	5	5.7	20	39	7
Constipation	31	35	3	12	3[4]	7	10	5	6.2	11	17	1
3rd sphincter rupture	21	22	2	1		2	17	7.1	7.2	2	15	1
Anal pain	9	8		1	1[5]		6	3.5	5.1	3	5	
Surg/Stoma	8	8			6		2	6.3	7	2	4	
Soiling	5	7	2	3	2			3.4	4.8	3	1	1
IBD	4	4		2			2	4.8	6.5	2	2	
Hemorrhoids	3	6	1	3	2[6]			4.3	6.3	2	1	
Anal atresia	2	2		1			1	6	7.5	2		
Fistula	2	2					2	4	5.5	1	1	
Fissure	1	2		1	15			2	8	1		
Pouchitis	1	1					1	8	7			1
Other	9	12	1	1	8	1	1	5.1	6.7	5	3	1
Average (SD)								5.1[b] (2.4)	6.1[b] (2.3)			
Total (%)	167	209	17 (8)	57 (27)	35 (17)	36 (16)	64 (31)			54 (35)	88 (57)	12 (8)

[1]Treatment as answered by patients, several treatments per patient possible; [2]Including all subgroups; [3]Sphincter repair; [4]Rectocele repair; [5]Fissurectomy; [6]Hemorroidectomy. [b]$P < 0.0001$.

Table 3 A comparison between the proposed therapeutic advice and followed therapy. A therapy can consist of more components

	All therapies (%)	Diet (%)	Medication (%)	Physiother (%)	Expectative (%)	Surgery (%)
Followed	130 (80)	7 (100)	54 (71)	32 (73)	31 (100)	36 (90)
Not followed	32 (20)	0	22 (29)	12 (27)	0	4 (10)
Total	162 (100)	7 (100)	76 (100)	44 (100)	31 (100)	40 (100)

physiotherapy were the least applied therapies (Table 3). Some disagreement between advised and followed therapy could be explained by the fact that patients considered fibres a diet instead of medication. Physiotherapy was advised in 44 patients (26%) and effectuated in 32 (73%). Ten years ago this was only respectively 18% and 67%[2]. Increasing interest in pelvic floor disorders and special training for physiotherapists has certainly contributed to the change in attitude towards physiotherapy[1,13]. Although therapeutic advice was given after AFE, actual improvement in symptoms is not necessarily caused by AFE. A placebo effect due to the referral to a specialized centre and the knowledge present in a 3rd referral centre may play a role. This is comparable with biofeedback studies for fecal incontinence, were the added value of the biofeedback was very difficult to separate from the received specialized care and treatment[13,14].

The examination was generally well tolerated, except in two patients with fistulas who reported the examination as painful. This was caused by local injection of hydrogen peroxide into the external fistula opening in order to visualize the fistula tract. It was remarkable that ten years ago only 13% of the females[2] and now 26% of the females preferred a female doctor. The larger number of referred Dutch ethnic minorities could not explain this.

Although the questionnaire was retrospective and has not been officially validated (we had used it before[2]), it has proven to be very useful. Questions about for instance surgery or 3-6 mo of physiotherapy could not easily be misunderstood. In patients treated in our own hospital follow up data were also obtained from the (electronic) patient files and no discrepancies were found with the answers provided by these patients.

Our treatment advice strategy is derived from clinical practice and the literature. In patients with fecal incontinence, regulating defecation and thickening of the fecal mass has proven to be effective and should always be tried first[15-17]. Biofeedback aimed at improving rectal sensation, recto-anal coordination and training external anal sphincter contraction is the next step and has a success rate varying from 40%-85% and is closely related to patient motivation[18]. Diarrhea should be properly diagnosed and treated before referring the patient for AFE since this overwhelming factor makes it impossible to establish the (possible) importance of anorectal causes. A rectal capacity between 60 and 100 mL will lead to fecal incontinence in 50% and < 60 mL in 100% of patients[12]; they will often need an enterostoma. Patients with fecal incontinence with a significant sphincter defect (> 25%) without severe neuropathy leading to atrophy can be identified as suitable candidates for a sphincter repair[1,19-21].

In our group of incontinent patients, only 12 (18%) ultimately underwent sphincter repair. Two patients were later referred for sacral neuromodulation elsewhere and eleven patients[22] were treated with SECCA® (radio frequent energy application to the external sphincter[23,24]).

Women who experienced a 3rd degree sphincter rupture are indicated for AFE, even without complaints. There in always some damage to the external anal sphincter and appropriate advice concerning defecation regulation, physiotherapy and possible future cesarean section can be discussed.

Most patients with constipation were referred for assessment of anismus/hyper tonic pelvic floor or rectocele. Generally AFE is not needed in these patients. Both anismus and a rectocele can be diagnosed by proper rectal examination[6,25,26]. When prolapse complaints dominate a defecography is indicated to demonstrate a possible enterocele as this can be corrected surgically. In patients with constipation, correction is not indicated in accidentally found intussusception since the obstructed defection will not improve[26-29]. AFE is indicated when (partial) colectomy is considered to be informed about the continence reserve. For patients with fistulas anal endosonography demonstrates the fistula tracts and anal manometry will establish the continence reserve[21,30-33]. In patients with soiling (anal secretion), medical history, good physical and rectal examination and an additional proctoscopy have proven be sufficient to establish a diagnosis[10], without the need for AFE, as was shown again in our patients. For patients with pain, AFE does not contribute[34]. Suspected discrete abnormalities e.g. an occult abscess, could not be demonstrated in our study as well. Sometimes a fissure is found in these patients, diagnosed on the basis of the medical history and rectal examination. In patients with a fissure, high pressures are usually found using manometry, but this does not alter therapy[35]. Only in those who where conservative measures have failed and will undergo surgery AFE seems indicated. In patients with haemorrhoids, anal manometry can also reveal high pressures and anal endosonography can demonstrate a thickened mucosa; however, these findings have no influence on therapy[36,37]. AFE is indicated in patients with an enterostoma when re-anastomosis is considered. In some rare disorders like anal atresia AFE can also be indicated to document anorectal problems and help choose a specific therapy.

In conclusion, feferral for AFE was indicated in 65%, communication and education to colleagues seems warranted. Indications are fecal incontinence without diarrhea, 3rd degree sphincter rupture, pre-operative for stoma or re-anastomosis, fistula, fissures or constipation. Anal ultrasound is always indicated in patients with fistula, anal manometry and rectal compliance when impaired continence reserve is suspected. Generally, in patients with constipation and soiling the medical history, physical examination and additional proctoscopy is sufficient and AFE is not necessary.

In 80% of patients, advice was followed. After AFE 92% benefited (35% of the patients improved and 57% was reassured). AFE is well tolerated. Women preferred a female doctor in 26% of cases.

COMMENTS

Background

Anorectal disorders like fecal incontinence, peri-anal fistula, pre-operative decisions for stoma are distressing and isolating conditions, with a large impact on quality of life. With restricted resources it is important to make a good selection of referrals for anorectal function evaluation, those patients who benefit most. In this study we established the indicated referrals our tertiary referral centre for patients with anorectal symptoms, the effect of the advised treatment and the discomfort of the tests.

Research frontiers

A clinical referral is indicated when disease can be demonstrated or excluded on the basic of anorectal function evaluation and when it has further therapeutical consequences. Which patients benefit the most from anorectal function tests (by reduction of symptoms or reassurance) is unclear.

Innovations and breakthroughs

Most studies mention anorectal function tests in relation to anorectal pathology and limit themselves to pre- and post-treatment results. Therefore it often remains unclear whether anorectal function evaluation leads to relevant findings or subsequent change of therapy. Literature concerning this issue is scarce. The aim of our study was to determine the indicated referrals to our tertiary center for patients with anorectal symptoms, the effect of advice on their complaints and the perceived discomfort for the patients during anorectal function evaluation.

Applications

It is very important to understand the usefulness of the anorectal function evaluation to provide referrals of those patients, which could benefit the most. Indications for anorectal function evaluation are fecal incontinence without diarrhea, 3rd degree sphincter rupture, pre-operative for stoma or re-anastomosis, fistula, fissures or constipation. Anal ultrasound is always indicated in patients with fistula, anal manometry. Rectal compliance is indicated when impaired continence reserve is suspected. Generally, in patients with constipation and soiling the medical history, physical examination and additional proctoscopy is sufficient and anorectal function evaluation is not necessary.

Terminology

Anorectal function evaluation consists of several tests: (1) anal manometry: establishes anal pressures; (2) rectal compliance: measures sensitivity and the volume of the rectum; (3) anal endosonography: visualizes possible defects or atrophy of the anal sphincter complex.

Peer review

The manuscript presents referral patterns for anal function investigation. Although the study has all limitations of a retrospective study, the authors provide valuable information from their experience as tertiary care center for patients with anorectal symptoms.

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RAPID COMMUNICATION

Changing spectrum of Budd-Chiari syndrome in India with special reference to non-surgical treatment

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Abstract

AIM: To evaluate patterns of obstruction, etiological spectrum and non-surgical treatment in patients with Budd-Chiari syndrome in India.

METHODS: Forty-nine consecutive cases of Budd-Chiari syndrome (BCS) were prospectively evaluated. All patients with refractory ascites or deteriorating liver function were, depending on morphology of inferior vena cava (IVC) and/or hepatic vein (HV) obstruction, triaged for radiological intervention, in addition to anticoagulation therapy. Asymptomatic patients, patients with diuretic-responsive ascites and stable liver function, and patients unwilling for surgical intervention were treated symptomatically with anticoagulation.

RESULTS: Mean duration of symptoms was 41.5 ± 11.2 (range = 1-240) mo. HV thrombosis (HVT) was present in 29 (59.1%), IVC thrombosis in eight (16.3%), membranous obstruction of IVC in two (4%) and both IVC-HV thrombosis in 10 (20.4%) cases. Of 35 cases tested for hypercoagulability, 27 (77.1%) were positive for one or more hypercoagulable states. Radiological intervention was technically successful in 37/38 (97.3%): IVC stenting in seven (18.9%), IVC balloon angioplasty in two (5.4%), combined IVC-HV stenting in two (5.4%), HV stenting in 11 (29.7%), transjugular intrahepatic portosystemic shunt (TIPS) in 13 (35.1%) and combined TIPS-IVC stenting in two (5.4%). Complications encountered in follow-up: death in five, re-stenosis of the stent in five (17.1%), hepatic encephalopathy in two and hepatocellular carcinoma in one patient. Of nine patients treated medically, two showed complete resolution of HVT.

CONCLUSION: In our series, HVT was the predominant cause of BCS. In the last five years with the availability of sophisticated tests for hypercoagulability, etiologies were defined in 85.7% of cases. Non-surgical management was successful in most cases.

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Key words: Budd-Chiari syndrome; Interventional radiology; Ascites; Hepatic vein thrombosis; Percutaneous transluminal angioplasty; Stent; Transjugular intrahepatic portosystemic shunt; Thrombophilia

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INTRODUCTION

Budd-Chiari syndrome (BCS) refers to hepatic venous outflow tract obstruction (HVOTO) starting from the level of small hepatic veins (HV) through large HV and inferior vena cava (IVC) to the junction of the IVC and right atrium[1-3]. This includes both hepatic vein thrombosis and IVC thrombosis or obliterative hepatocavopathy[4,5].

Clinical studies on BCS in India date back to the 1970s[6-10]; following which, there are many case reports and case studies from India, concentrating on clinical spectrum, on underlying etiology, on diagnostic modalities and/or on various treatment strategies[11-47]. In contrast to the western world, in the Indian series, there is a striking predominance of IVC obstruction (mainly from membrane or web) or combined IVC-HV obstruction rather than HV obstruction alone[12-15]. Tumors, pregnancy, oral contraceptive pills (OCP) and infections were proposed as predominant underlying etiologies, hypercoagulable states were uncommon, and idiopathic cases were the most common[9,12-21,42]. In the series of cases during the last 10 years, various hypercoagulable states are being increasingly described as etiologies of BCS[24-26,28-30,46,47].

Obstruction of the IVC, either thrombotic or non-thrombotic, was considered to be a major cause of BCS in Asia[5]. Two-thirds of IVC obstruction cases leading to BCS are due to membranous obstruction[11]. Datta *et al*[6] reported 40 cases of membranous obstruction of IVC (MOVC) while Victor *et al*[11] reported 17 cases

of membranous obstruction. Though MOVC and membranous obstruction of HV was initially thought to be congenital, Okuda demonstrated these lesions to be an after-effect of thrombosis in the IVC or HV which were organised over a period of time[5]. Similar cases of have been reported from Nepal[53]. Originally, it was perceived that MOVC and membranous obstruction of HV are important causes of BCS which is different from the West. Over the years, many prothrombotic states leading to BCS have been described in India[28,47]. In an elegant review, Valla has described the level of HVOTO, the nature of obstructive lesions, presentation, course of disease and causes that are comparable in Indian and western literature[4]. A few differences in etiologies were noticed between India and the West i.e. peripartum occurrence of BCS was common in India while oral contraceptive use was commonly implicated in the West.

The majority of patients with BCS present with a chronic course, while only a small number of patients present with acute or fulminant forms[6-22]. BCS is mostly encountered in the adult population and considered uncommon in children; when seen in children, the clinical presentation is similar[13]. Anti-thrombotic drugs and anticoagulants form the mainstay in the treatment of acute and chronic BCS. Percutaneous balloon angioplasty for membranous obstruction of the IVC and hepatic vein has also been used successfully in the treatment of BCS. Although TIPS has gained popularity in the treatment of BCS, it has rarely been performed in India, with only one case being reported[41]. Furthermore, there are only a few reports on the use of covered TIPS in BCS. In this report, we evaluated patterns of obstruction, aetiological spectrum and non-surgical treatment of BCS in patients.

MATERIALS AND METHODS

In last seven years (during the study period of 1999 to 2005), all the consecutive cases of BCS were prospectively evaluated. Diagnosis of BCS was based on angiographic evidence of HVOTO (i.e. obstruction of IVC and/or HV) and/or histological evidence of BCS (sinusoidal dilation with centrilobular congestion with variable amount of pericentral hepatocyte necrosis and pericentral fibrosis).

The protocol for evaluation of BCS in our unit is shown in Figure 1. BCS was suspected in the following situations: patients with ascites of high serum-ascitic fluid albumin gradient (> 1.1) and with low cell count (< 250 cells/cm^2); patients with ascites and/or back veins in the presence of hepatomegaly and/or right upper quadrant pain; patients with refractory ascites, patients with acute liver failure with hepatomegaly and/or ascites; patients with a known hypercoagulable state who showed evidence of liver involvement (on clinical examination, biochemistry or imaging); and patients with unexplained chronic liver disease (when work-up for alcoholic, drug-induced, viral, metabolic or autoimmune liver disease were negative). All such patients (suspected BCS) were subjected to ultrasonography of the abdomen and Doppler studies with special emphasis on IVC, HV and splanchnic venous system. If HVOTO was confirmed, patients were subjected to inferior vena cavogram and hepatic venogram

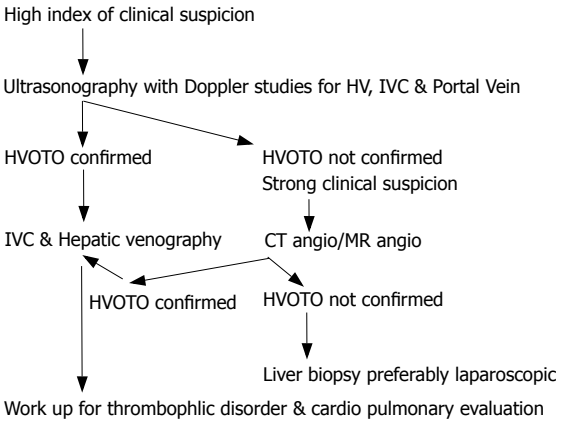


Figure 1 Evaluation of patients with Budd Chiari syndrome.

(*via* transfemoral, transjugular and/or transhepatic route) to define the site and morphology of the obstruction. However, if ultrasonography/Doppler was negative or ambiguous for HVOTO, computerised tomography (CT) angiography or magnetic resonance (MR) angiography (if CT angiography was contraindicated) was performed. If CT angiography or MR angiography showed HVOTO, these patients were subjected to catheter venography. If computerised tomography with angiography or magnetic resonance angiography was negative, these patients were subjected to liver biopsy (either *via* percutaneous or transjugular route depending on clinical status) to define BCS and rule out other diseases like veno-occlusive disease. All patients with suspected BCS were subjected to chest x-ray, electrocardiogram and 2-dimensional echo of the heart to rule out cardiac etiology. Liver histology was performed whenever possible to confirm the diagnosis and to define the presence of cirrhosis. Upper gastrointestinal endoscopy was performed in all cases of BCS to determine the presence of varices.

All the patients with BCS were subjected to tests available for hypercoagulable state before starting any treatment. In the initial two years of the study period, tests for protein C, protein S and antithrombin Ⅲ levels in addition to work-up for myeloproliferative disease and paroxysmal nocturnal haemoglobinuria (PNH) were performed. During the last five years of the study period, tests for protein C, protein S and antithrombin Ⅲ levels (corrections for liver dysfunction were done)[48], serum homocysteine levels, factor V Leiden, prothrombin gene 20210 and MTHFR gene mutations, lupus anticoagulant, anticardiolipin and antiphospholipid antibodies, tests for PNH (Sucrose lysis and Ham tests), complete blood counts and bone marrow histopathology/cytogenetic studies (whenever feasible) for myeloproliferative disorder were performed. Imaging (ultrasonography, computerised tomography and/or magnetic resonance imaging of abdomen), serological markers (including tumor markers and tests for amoebiasis or echinococcus) and/or histology were done to identify underlying etiology for BCS as and when required. All the female patients were subjected to a urine test for pregnancy and were questioned regarding use of oral contraceptive pills.

All patients with refractory ascites (RA) or deteriorating

Table 1 Clinical parameters in 49 patients with BCS

Clinical features	% of Patients
Ascites	86
Distended abdominal wall veins & back veins	28
Jaundice	20
Splenomegaly	20
Pedal edema	12
Upper GI bleeding	8
Infertility	6
Fever	8
Hepatic encephalopathy	4
Hydrothorax	6
Vericocity	4
Asymptomatic	6
Previous episode of thrombosis	8
Family history of thrombotic event	2
Previous antituberculous treatment	12
Mean duration of symptoms prior to diagnosis	41.5 + 11.2 mo (range 1-240 mo)
Types of presentations	
Asymptomatic	6
Fulminant	8
Acute	41
Chronic	42

Patients were classified as per definitions in reference number 4

Table 2 Laboratory parameters in 49 patients with BCS

Parameter	Value	Range
Sr. Bilirubin mg%	1.49 ± 1.2	0.2-5.6
Sr. ALT Iu/mL	53.4 ± 13.2	17-424
Sr. AST Iu/Ml	61.6 ± 15.6	16-724
Sr. AlkPO4 Iu/mL	159.6 ± 35	91-313
Sr. GGPT	109.6 ± 1.2	18-417
Sr. Albumin g/Dl	3.44 ± 1.2	1-4.8
Increase in prothrombin time	3.5 ± 1.1	1-10 s

All the patients were negative for serology of Hepatitis B, C & HIV.

liver function (presence of hepatic encephalopathy (HE) or jaundice, serum bilirubin > 2 mg/dL, serum albumin < 3 gm/dL and/or international standardised ratio of prothrombin time (INR) > 2), depending on morphology of IVC and/or HV obstruction, were triaged for either IVC stenting, HV stenting, TIPS or in combination: (1) For supra-hepatic IVC block with patent HV: IVC balloon angioplasty with self-expandable metallic stent (SEMS) placement; (2) For membranous obstruction of inferior vena cava (MOVC): IVC balloon angioplasty; (3) For juxta-hepatic IVC block with patent HV: IVC balloon angioplasty with SEMS placement; (4) For juxta-hepatic IVC block and short-segment HV block (< 3 cm) involving all three HV or two major HV: IVC balloon angioplasty with SEMS placement and HV balloon angioplasty with SEMS placement; (5) For juxta-hepatic IVC block and long-segment HV block (> 3 cm): IVC balloon angioplasty with SEMS followed by TIPS placement; (6) For short-segment HV block (< 3 cm) involving all three or two major HV: HV balloon angioplasty with SEMS placement; (7) For long-segment HV block (> 3 cm) involving all three or two major HV: TIPS placement. In addition to symptomatic therapy, all these patients were started on anticoagulation following radiological intervention with the aim of keeping INR 2-3. Asymptomatic patients, patients with diuretic-responsive ascites, patients with stable liver function or patients who were not willing to undergo radiological intervention were subjected to anticoagulation to keep INR 2-3 in addition to symptomatic therapy (including low-salt diet, diuretic therapy and/or beta-blockers as needed). Peritoneo-venous shunting was offered to patients with RA who were not candidates for any intervention.

Follow-up was done on the 3rd d, at the end of the 1st mo, every 3rd mo for the 1st year and every 6th mo thereafter. During each follow-up visit, clinical, biochemical and ultrasonography/Doppler evaluations were done. Patients with TIPS or SEMS dysfunction were managed by repeat radiological interventions.

For patients undergoing TIPS, the following outcome measures were used: (a) technical success: creation of a channel by stent between hepatic vein and portal vein to reduce the portosystemic pressure gradient (PPG) to less than 12 mmHg; (b) TIPS dysfunction: 50% decrease in portal vein blood flow velocity on Doppler, angiographic evidence of stent stenosis (> 50% reduction in diameter) or increase in PPG > 12 mmHg in presence of recurrent ascites/hepatic hydrothorax or gastrointestinal bleeding; and (c) primary patency: duration of continuous TIPS patency without re-intervention. Comparison between uncovered TIPS and covered TIPS was done using chi square test. Institutional review board permission was obtained.

RESULTS

During the study period, 49 patients (mean age = 34.3 ± 6.5 years; age range = 1-57 years; male:female ratio = 24:25) were included in the study. In our series there were only two children below the age of 18 years. One child at the age of one year presented with rapidly accumulating ascites, the other child at the age of 11 had refractory ascites for 5 mo.

Clinical presentation and biochemical features of these patients are shown in Tables 1 and 2.

Ultrasonography with Doppler studies was diagnostic in 80% of patients. The remaining 20% of patients required CT/MR angiography for diagnosis. Liver histology was done in 28 (57.1%) cases, of which 14 (50% of 28 cases) were cirrhotic.

Morphology of hepatic venous outflow tract obstruction was short segment hepatic vein thrombosis in 13 patients, long segment in 16 patients, supra hepatic IVC in three, juxta hepatic IVC in five, IVC plus short segment hepatic vein in six and long segment hepatic vein in four. Associated portal vein thrombosis was seen in two (4%) and superior mesenteric vein thrombosis was seen in one (2%) patient. Features of caudate lobe hypertrophy and extrinsic compression of IVC (on imaging and/or IHV) was seen in 30 (61.2%) cases. One MOVC case had past imaging (on CTA) evidence of IVCT, which was done to rule out intraabdominal malignancy as a cause of femoral vein thrombosis and was negative for the same. At that time, IVCT patient was asymptomatic. Four years later this patient presented with symptomatic MOVC and was found to have factor V Leiden mutation.

Etiological spectrum in 35 patients who had undergone

all tests for thrombophilia work up was Polycythmia vera 3 (8.5%), Protein C deficiency two (6%), Protein S deficiency one (3%), Hyper homocysteinemia two (6%), Anti thrombin Ⅲ deficiency three (9%), Anti cardiolipin antibodies five (14%), Factor V laden mutation four (11.4), Lupus anti-coagulant two (6%), PNH one (3%), Multiple abnormalities four (11%), Renal Cell Carcinoma one (3%), IVC leiomyoma one (3%), Acute myeloid leukemia one (3%), Idiopathic six (19%). None of the patients was pregnant or taking OCP.

Radiological interventions done in 38 patients were as follows: IVC stenting eight (20%), IVC balloon angiography two (5%), Combined IVC Hepatic Vein stenting two (5%), Hepatic vein stenting 11 (30%), TIPS 15 (40%), and IVC stenting with TIPS two (5%).

In one patient with supra-hepatic IVC block with refractory ascites and deteriorating liver function, attempted IVC stenting failed; he was then lost to follow-up. In the radiological intervention group, technical success was achieved in 97.3% (37/38 cases). One (2.7%) patient had a technical complication of post-TIPS (uncovered TIPS) peritoneal haemorrhage, to which the patient succumbed. Another patient (HV stenting) with acute myeloid leukemia succumbed within 1 mo of follow-up. Complete resolution of ascites or improvement in liver function was seen within 3 mo of follow-up in the remaining 35 cases. During further follow-up (mean period = 24.5 ± 12.5 mo, range = 3-84 mo) of these 35 cases, mortality was 8.5% (3/35 cases): causes of death were liver-related in one case [resistant HE (covered TIPS-related)] and non-liver related in two cases [one with intracranial bleeding (anticoagulation therapy related, one in IVC-HV stenting group) and one with metastatic renal cell carcinoma (one with IVC stenting)]. Details of other complications encountered during follow-up are as follows: (a) Episodes of HE responsive to medical treatment were seen in two post-TIPS (both covered TIPS) cases (5.7%). (b) Re-occlusion of stent was seen in six (17.1%) cases (one at 1-mo, two at 3-mo, two at 6-mo and one at 1-year follow-up) of which one had IVC stent, two had HV stent, two had uncovered TIPS and one had covered TIPS, radiological re-intervention (balloon dilatation) was possible in all cases. During further follow-up, in the event of stent occlusion, one patient with uncovered TIPS was offered covered TIPS and one patient with HV stent underwent balloon angioplasty again. (c) Hepatocellular carcinoma (HCC) was encountered at 2-year follow-up in one (2.8%) patient with MOVC, who had undergone IVC balloon angioplasty and was treated three times with trans-arterial chemo-embolisation during further five years follow-up. During follow-up of these 35 cases, only two patients (of HV stenting) were lost to follow-up after 6 mo.

On evaluating the patient subset with TIPS treatment, technical success was achieved in all 15 (100%) patients (five uncovered TIPS and 10 covered TIPS), procedural complication (uncovered TIPS) was seen in one (6.6%) patient, total mortality (one in uncovered TIPS and one in covered TIPS) was 13.3% (two patients), re-occlusion rate was 23% (one with covered and two with uncovered TIPS) in 13 survivors, primary patency rate at 6 mo for uncovered TIPS was 50% and for covered TIPS was 88.9%, and occurrence of new-onset HE was in 20% (3 patients of covered TIPS). There was a statistically significant difference ($P < 0.05$) in primary patency rate between covered and uncovered TIPS.

Eleven (22.4%) patients [all patients had stable liver functions, three cases were asymptomatic cases and three cases had RA; five patients with HVT (two short-segment and three long-segment) and six patients with IVCT-HVT (two long-segment HVT and four with short-segment HVT)] were treated with anticoagulation in addition to symptomatic therapy (mean duration of follow-up = 28.4 ± 10.8 mo, range = 1-74 mo). Two patients with RA, who were not willing to undergo radiological intervention, underwent placement of PVS. Of these, one patient had blockage of PV shunt and died of sepsis after five years; the other patient was all right at four years follow-up. Of the remaining nine cases, one patient with RA (unwilling to undergo any intervention) died of progressive liver failure after 3 mo; two patients (one at 6-mo and one at 1-year follow-up) with HVT showed complete resolution of ascites, normalisation of liver function tests and re-canalisation of HV on USG-D (repeat IHV was not done); three asymptomatic patients remained asymptomatic until 6-mo follow-up, but were lost to follow-up then; three patients were lost to follow-up after 1 mo.

Overall, in 42 cases who completed at least 6 mo of follow-up, mortality was seen in 4/42 cases (9.5%), and evidence of HCC in 1/42 (2.3%) cases.

DISCUSSION

Our study is distinct from previous Indian reports in many aspects. It showed predominance of HV thrombosis and identification of etiologies in more than three out of four of cases, the majority being hypercoagulable states. To the best of our knowledge, IVC leiomyoma and acute myeloid leukemia are being reported as etiologies for the first time in India. Also, cases showing transition from IVC thrombosis to MOVC and development of hepatocellular carcinoma in MOVC are well documented. For the first time in India, the experience of using covered TIPS in BCS is presented.

In our series, HV thrombosis represented the majority of cases (59.1%), which were followed in decreasing order by combined IVCT/ HV thrombosis (20.4%), IVC thrombosis (16.3%) and MOVC (4%). All previous Indian series, except two[29,32], describe the predominance of IVC up to 79.2%[7,9,11-13,15,30,39,40,42] or IVC-HV obstruction up to 57.7%[14,24,25,35]; HV involvement was described in 0%-32% of cases in these series[7,9,11-15,24,25,30,35,39,40,42]. Of the two distinct previous series, one (total 53 cases) showed similar frequency of HV (35.8%), IVC (33.9%) and combined IVC-HV involvement (30.1%)[29]; whereas the other series involving only chronic BCS showed predominance of HV (45.9%) followed by combined IVC-HV (29.7%) and IVC involvement (24.3%)[32]. In the western countries, hepatic vein thrombosis remains responsible for majority of BCS cases, IVC obstruction is rarely found. In Asian and African countries, more common is IVC involvement. In Japan, over span of last 30 years, there is change in the spectrum i.e. marked decrease in cases of IVC

obstruction[5,49-52]. Is similar trend is following in India or this is just reflection of selection bias at tertiary care centre? For definite answer, reports from all over the country will be needed in support.

Previously, MOVC was thought to be the predominant cause of IVC involvement (20.4%-58.6%) and of BCS in India[6,7,11-15,17,29,35,39], as well as in Asian countries[49,50,53]. In two recent Indian studies, MOVC was present in 0% and 17.2%, while IVC involvement was seen in 56.2% and 62% cases, respectively[24,40]. In our series, IVC involvement in the majority of cases was due to IVC thrombosis. Previously, MOVC and IVC thrombosis were considered to be idiopathic in origin by most Indian and Asian workers[5,6,11,13-15,17,35,49,50,53,54]; but this view has been challenged recently in a few reports[22,29,30,42,55-58]. In cases of IVC thrombosis in our study, the etiology was identified in 50%. In our series, one of two MOVC cases had a hypercoagulable state and in that case, we were able to demonstrate evolution of IVC thrombosis to MOVC. Transition from IVC thrombosis to MOVC is well described in worldwide literature[5,59,60]. The other patient with MOVC in our study developed hepatocellular carcinoma, without any other predisposing factors, such as: hepatitis B, hepatitis C or alcohol. Reports of hepatocellular carcinoma developing in MOVC are rare in Indian literature[7,12-15]. However, such an association is commonly described in most other countries[5,49,50,53,61-64].

In our study, with the application of all commercially available hypercoagulability tests, an underlying etiology could be defined in 85.7% of cases, the majority having a hypercoagulable state. This was in contrast to the initial study period (before 2001), during which few tests were performed, where etiology was evident only in 28.5% cases. None of our patients were pregnant, taking oral contraceptive pills, in a post-partum state, had abdominal trauma, abdominal surgery, amoebic liver abscess, pyogenic liver abscess, hydatid cyst, hepatic tuberculosis or filariasis; these were the predominant causes in the previous Indian studies[9,12-20,23,42]. Previous Indian series (before the year 2001) have shown identification of underlying etiology in 12%-50%, of which hypercoagulable states were uncommon[9,12-15,42]. In the last decade, there are many reports identifying one or another hypercoagulable state[21,22,24-28,46,47]. Recently, application of more tests has defined hypercoagulability in up to 59% of cases[29,30]. In addition to tests performed in our study, studying haemopoietic stem cell defects in bone marrow may further identify occult MPD, as previously shown in two Indian studies[26,46] and in many western studies[4,64]. As in our series, multiple co-existing etiologies have recently been described throughout the world[29,65]. Our work was in accordance with the majority of western reports, where identification of etiology is more than 75%[2], and at a few centres more than 90%[64-66]. Changes in the etiological spectrum probably represents the effect of availability and application of tests for hypercoagulability.

Renal cell carcinoma, which was present in three cases in our study, was rarely reported in the past[15]. Likewise, IVC leiomyoma and acute myeloid leukemia as underlying etiologies are described for the first time in Indian literature. Other malignancies described in the past include hepatocellular carcinoma, adrenal tumor, Wilm's tumor, cholangiocarcinoma and chronic lymphoid leukemia[9,13-15,39,42].

In a few examples, medical treatment only was shown to be effective in a subset of patients with stable liver function, diuretic-responsive ascites and asymptomatic presentation[2,67,68].

Few reports have shown the beneficial results of surgery in selected BCS patients with low operative mortality up to 5%, high long-term assisted patency rate of more than 90% and five-year survival rates more than 75%[2,69-72]. Poor results of surgery in patients with liver dysfunction; anatomical difficulties causing success rates as low as 30%; higher post-operative complications and mortality rates (more than 20%); high re-stenosis rates (around 30%) requiring surgical revisions in a minority (around 10%); and poor survival rates as low as 57% at five years have been described[1,3,4,70,73,74]. Surgical treatment has also failed to show a favorable effect in two multivariate analyses[68,75], but was successful in one other study[76]. In most Indian reports, while surgery for IVC involvement has shown fair results[34,36]; surgical treatments for HV occlusion have shown poor results[12,38]. These were the driving force in deciding not to offer surgical treatment to our patients.

Radiological interventions for both IVC and HV achieved technical success rates of more than 90% and long-term patency rates of more than 80%; also radiological re-interventions are usually successful[1,69,77-80]. In our series, radiological intervention protocols according to morphology of the obstruction yielded good results. Previous Indian series have shown the efficacy of balloon angioplasty of IVC with or without stent placement [11-13,37,39,40,43-45], with a re-stenosis rate ranging from 16.6% to 28.5%[11,39]. Balloon angioplasty of HV thrombosis has shown variable results throughout the world, restricting its use to short-segment HV thrombosis only[4,77]. In India, experience with HV balloon angioplasty is rare[12,37]. Patients with combined IVC-HV blockages, which are considered difficult to treat, were treated with balloon angioplasty followed by surgical portosystemic shunting in previous Indian studies, but post-operative complications and mortality were prominent[12]. In a recent study from China, a two-stage approach was recommended, the first stage being IVC stenting followed by another session of HV stenting[77]. In our work, we successfully performed balloon angioplasty with stent placement in IVC followed by either TIPS or HV balloon angioplasty with stent placement in a single session.

In the last decade, the use of TIPS in BCS is increasingly described in the world literature[81-94]. In cases with severe liver dysfunction requiring liver transplantation, TIPS used as an interim bridge to transplantation, can improve the situation dramatically[4]. In most studies, TIPS was used as rescue therapy in patients with failed or re-occluded radiological or surgical interventions or was used as a bridge to liver transplantation. Difficulty with TIPS may be associated splanchnic venous thrombosis, which can be successfully tackled by radiological interventions in the same session[89]. Technical success for TIPS ranges from 75% to 100% in various works[81,84,88,90,93]. The problem

of TIPS dysfunction (present in 40% to 75% if followed up for more than two years[80,82,84,90,91,93]) necessitates re-intervention in up to 70% of cases[1,87-89,91], giving a revision rate of 1.4 revisions per patient[91]. TIPS related complications occur in less than 20% of patients[91]. TIPS provided a survival rate of 85% at four years in one series and 74% at five years in another series[82,91]. Covered TIPS in BCS has shown lower TIPS dysfunction rate (33% *vs* 87% in uncovered group) and higher primary patency rate at 1-year (67% *vs* 19% in uncovered group) in one recent series comprising nine patients in a covered TIPS group[94]. Another series using covered TIPS in eight patients showed there was no need for revisions during almost one year of follow-up[91]. In our study, TIPS was used as a primary treatment in the subset of the patients with long-segment HV thrombosis with or without IVCT. During the study period (mean follow-up of more than two years), the technical success rate was 100%; procedural complication rate was 6.6% (post-TIPS peritoneal haemorrhage); total mortality rate was 13.3%, re-occlusion rate was 23%; primary patency rate for uncovered TIPS was 50% and for covered TIPS was 88.9%; and new-onset hepatic encephalopathy rate was 20%. All the re-occlusions were tackled radiologically. Our study, although with a small number of patients, showed promising results with TIPS as the primary therapy in BCS patients. Covered TIPS was better than uncovered TIPS in terms of long-term patency.

COMMENTS

Background

Budd-Chiari syndromes (BCS) has been commonly described in India. It was always considered that the spectrum of BCS in India is different from that of the west.

Research Frontiers

In this study we discussed the changing spectrum of BCS in India which is almost comparable to the west.

Innovation and Breakthroughs

Radiological interventions including TIPS with a covered stent has been useful in managing these patients with good long term efficacy.

Application

This study will help to improve the management of patients with BCS in India.

Terminology

BCS is an uncommon condition induced by thrombotic or nonthrombotic obstruction to hepatic venous outflow. It was first described by Budd in 1845 and Chiari later added the first pathologic description in 1899.

Peer review

It's an excellent overview of BCS in India, showing a change in the aetiology towards hypercoagulopathic diseases. They authors could as well demonstrate the effectiveness of TIPS, especially when using covered stents.

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RAPID COMMUNICATION

Child-Pugh-Turcott versus Meld score for predicting survival in a retrospective cohort of black African cirrhotic patients

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Abstract

AIM: To compare the performance of the Child-Pugh-Turcott (CPT) score to that of the model for end-stage liver disease (MELD) score in predicting survival of a retrospective cohort of 172 Black African patients with cirrhosis on a short and mid-term basis.

METHODS: Univariate and multivariate (Cox model) analyses were used to identify factors related to mortality. Relationship between the two scores was appreciated by calculating the correlation coefficient. The Kaplan Meier method and the log rank test were used to elaborate and compare survival respectively. The Areas Under the Curves were used to compare the performance between scores at 3, 6 and 12 mo.

RESULTS: The study population comprised 172 patients, of which 68.9% were male. The mean age of the patient was 47.5 ± 13 years. Hepatitis B virus infection was the cause of cirrhosis in 70% of the cases. The overall mortality was 31.4% over 11 years of follow up. Independent factors significantly associated with mortality were: CPT score (HR = 3.3, 95% CI [1.7-6.2]) (P < 0.001) (stage C *vs* stage A-B); Serum creatine (HR = 2.5, 95% CI [1.4-4.3]) (P = 0.001) (Serum creatine > 1.5 mg/dL versus serum creatine < 1.5 mg/dL); MELD score (HR = 2.9, 95% CI [1.63-5.21]) (P < 0.001) (MELD > 21 *vs* MELD < 21). The area under the curves (AUC) that predict survival was 0.72 and 0.75 at 3 mo (P = 0.68), 0.64 and 0.62 at 6 mo (P = 0.67), 0.69 and 0.64 at 12 mo (P = 0.38) respectively for the CPT score and the MELD score.

CONCLUSION: The CPT score displays the same prognostic significance as does the MELD score in black African patients with cirrhosis. Moreover, its handling appears less cumbersome in clinical practice as compared to the latter.

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Key words: Model for end-stage liver disease score; Child score; Cirrhosis; Black African; Survival

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INTRODUCTION

The Child-Pugh-Turcotte (CPT) score has usually been used to assess the prognosis of patients with cirrhosis[1], since it is related to the severity of the liver disease.

The detrimental effect of renal failure on survival of patients with cirrhosis is widely acknowledged[2-6]. The prevalence of renal failure in cirrhotic patients varied between 7%-65%[7-9]. About 20% of the cirrhotic patients presenting with ascites were likely to display an acute pre-renal failure. This value has been reported to exceed 50% when end-stage liver disease is present[10].

The model for end-stage liver disease (MELD) score has recently been developed to better rationalize liver graft allocation. Indeed liver graft allocation has been so far performed according to the severity of the liver failure as determined by the CPT score plus the time spent on the waiting list. Since the first transplanted have almost been the first on the waiting list and knowing that they did not necessary display the most severe disease, such an approach has been subjected to debate[11-17]. The MELD score has been elaborated using three independent prognostic factors: creatine, International Normalized Ratio (INR) and bilirubin. In a study in 311 patients on the waiting list, the MELD score has been found adequate to predict mortality[12-20]. The MELD score has been thus far used in the USA for liver graft allocation in place of the CPT score.

Several studies have been performed to compare the performance of the two scores in predicting survival in patients with cirrhosis[21-33]. However such studies have yielded conflicting results[23, 24, 28, 30-33].

The aim of the present study was to compare the performance of the MELD score to that of the CPT score to predict survival in a retrospective cohort of 172 black African patients with cirrhosis.

MATERIALS AND METHODS

Type of study

A retrospective analysis of a cohort of black African patients with cirrhosis has been undertaken. Patients have been recruited from January 1, 1991 to December 31, 2001 (11 years) at the hepatology unit of the University hospital of Yopougon (Abidjan), one of the three tertiaries hospitals of the capital, admitting and hospitalising at least 650 patients a year for a total capacity of 20 beds.

Patients

In our daily practice, all the patients who were diagnosed as having cirrhosis were systematically hospitalised, because most of them were seen at a very late stage of the disease. Of 307 patients recruited over this study period, 135 have been excluded because of missing data. 172 patients have, therefore, been considered for analysis.

Methods

Data of the patients such as, age, gender, motives of admission, aetiology of cirrhosis, serum creatine, CPT score and the MELD score, date of first symptoms of decompensated cirrhosis, date of first admission, date of last visit for the patients lost to follow up or still alive, the date of death for deceased patients have been all collected using a standardized collection sheet.

Definitions

The CPT score has been widely described elsewhere. It has been elaborated by using five (5) parameters: hepatic encephalopathy, ascite, prothrombin in percentage, serum albumin in g/L and bilirubin in mg/L.

The MELD score: It has been elaborated by using the Malinchoc formula which comprises 3 parameters: INR, bilirubin (mg/dL) and creatine (mg/dL). This formula is as follow: [9.57 × Loge creatine (mg/dL) + 3.78 × Loge bilirubin (mg/dL) + 11.20 × Loge INR + 6.43].

Time of follow up: It corresponds to the time between the date of first admission for cirrhosis and the date of last visit or the date of death for deceased patients.

Time of survival: It corresponds to the time between the date of the first symptoms of decompensated cirrhosis as reported by the patient and the date of last visit for the patients lost to follow up or still alive or the date of death for the deceased patients.

Statistical analysis

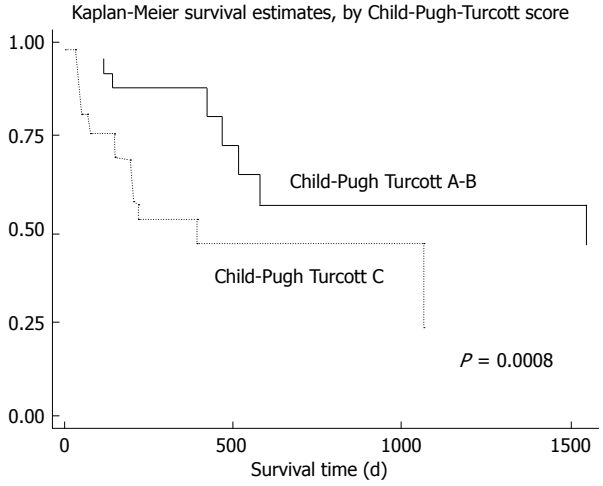
By univariate analysis, relationship between mortality and the following parameters has been assessed: age, gender, aetiology of cirrhosis, serum creatine, CPT score and the MELD score. The Student t-test or the Mann-Whitney test was used for quantitative parameters and the chi square test or the Fischer exact test for qualitative parameters. A multivariate analysis has been performed using a Cox model in a forward selection manner. Two models have been elaborated subsequently: the first has included all the parameters used in the univariate analysis with the exception of serum creatine. The second model has included all the parameters used in the univariate analysis at the exception of the MELD score. Survival has been elaborated using the Kaplan Meier method. The log rank test has been used for comparison of survival between groups. Correlation between the two scores has been evaluated. The ROC curve has been used to compare performance between scores.

Table 1 Characteristics of the study population

Male (%)	120/172 (69.8)
Mean age (yr)	47.5 ± 13.54
Child-Pugh-Turcott score (%)	
A	20 (11.6)
B	54 (31.4)
C	98 (57)
Serum creatine (mg/dL) (%)	
⩽ 1.5	132 (76.7)
> 1.5	40 (23.3)
MELD score (mean ± SD)	20.9 ± 10.87
MELD: Median (extreme)	19.5 [3-66]
Etiology (%)	
Virus B	78 (45.3)
Virus C	17 (10)
Both alcohol and virus B or C	41 (23.8)
Unknown	36 (20.9)

RESULTS

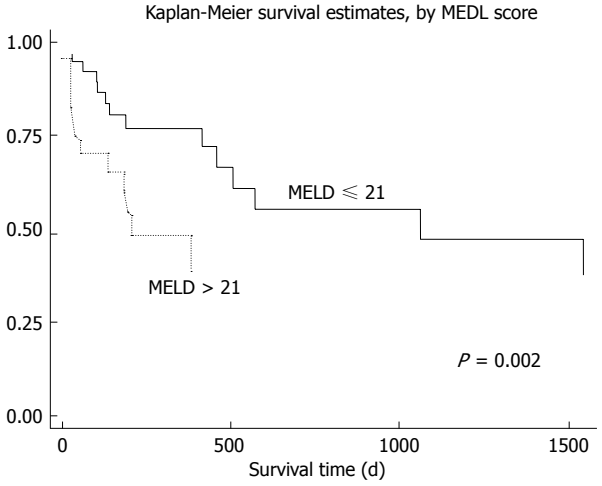
The different motives of admission of patients with cirrhosis were: ascites and oedema of the lower limbs (68.6%), jaundice (25.2%), haemorrhage of the digestive tract (15.1%) and hepatic encephalopathy. The study population comprised 172 patients, of which 69.8% were male. The mean age of the patients was 47.5 ± 13.54 years. 57% of the patients were with a CPT score stage C, 23.3% had renal failure and the mean MELD score was 20.9 ± 10.87. Hepatitis B was the main cause of cirrhosis in 45.3% of the case (Table 1). Over the study period (11 years), 54 out of 172 patients (31.4%) have died, of which 22 during the first three months and 32 the first 6 mo. The median and the mean duration of follow up were 206 and 226.6 + 41.6 d, respectively. By univariate analysis, the following parameters have appeared to significantly influence mortality: age > 48 years old ($P < 0.023$); male gender ($P < 0.003$); CPT score stage C ($P < 0.016$); serum creatine > 1.5 mg/dL ($P < 0.001$) and MELD score over 21 ($P < 0.03$). Indeed, the mean CPT score has appeared significantly less elevated in living cirrhotics as compared to those who have died during the study period (10.2 ± 0.28 *vs* 11.3 ± 0.33, respectively; $P = 0.02$), as it was with the MELD score (19.4 ± 0.83 *vs* 24.2 ± 1.85, $P = 0.007$, respectively). Moreover mortality increased proportionally to the level



Probability of survival (95% CI):

	Child-Pugh-Turcott A-B (%)	Child-Pugh-Turcott C (%)
-3 mo	100	75.1 (64.5-82.9)
-6 mo	87.3 (73.9-94.1)	68.6 (57-77.7)
-12 mo	87.3 (73.9-94.1)	52.1 (38.8-63.9)
-24 mo	56.0 (35.9-72)	45.6 (31.4-58.8)

Figure 1 Survival according to the CPT score.



Probability of survival (95% CI):

	MELD ⩽ 21[1] (%)	MELD > 21[1] (%)
-3 mo	93.3 (85.6-97.0)	71.2 (57.2-81.4)
-6 mo	81.7 (70.9-88.8)	66.1 (51.3-77.4)
-12 mo	78.0 (66.2-86.1)	49.0 (33.1-63.1)
-24 mo	56.3 (40.4–69.4)	39.2 (22.3-55.7)

Figure 2 Survival according to the MELD score. [1]Through value of the MELD score significantly associated with mortality.

Table 2 Relationship between mortality and parameters

Parameters	Mortality		
	Univariate analysis		Multivariate analysis
	Death (%)	*P*	Hazard ratio (95% CI); *P*
Age (yr)[1]			
⩽ 48	23.90	0.023	
> 48	40.00		
Gender			
Female	15.40	0.003	
Male	38.30		1.8 [0.96 - 3.24]; *P* = 0.07
Child-Pugh-Turcott			
A-B	21.60	0.016	
C	38.80		3.3 [1.7 - 6.2]; *P* < 0.001
Serum creatine (mg/dL)			
⩽ 1.5	24.20	< 0.001	
> 1.5	55.00		2.5 [1.4 - 4.3]; *P* = 0.001
MELD[2]			
⩽ 21	25.50	0.030	
> 21	41.90		2.9 [1.63 - 5.21]; *P* < 0.001
Aetiology			
Virus B or C	32.90	0.670	
Both alcohol and virus[3]	40.20		
Unknown	36.80		

[1]Median age; [2]Through value of the MELD score significantly associated with mortality; [3]Aetiology both virus and alcohol.

Table 3 Stepwise evaluation to find out the optimal cutoff point for both CPT and MELD score (%)

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
CPT score				
8	96.30	20.30	35.60	92.30
9	74.00	33.00	33.60	73.60
10[1]	70.40	49.20	38.80	78.40
11	66.70	52.60	39.10	77.50
12	59.30	57.60	39.00	75.60
MELD score				
19	55.60	52.50	34.90	72.10
20	55.60	57.60	37.50	73.90
21[2]	48.20	69.50	41.20	74.60
22	44.00	69.50	40.00	73.20
23	44.40	76.30	46.20	75.00

[1]Optimal cutoff point for CPT (Child-Pugh-Turcott) score; [2]Optimal cutoff point for MELD (Model for End-stage Liver Disease) score.

of the CPT score (0% for stage A, 29.6% for stage B and 38,8% for stage C; $P = 0.001$), as it did with the MELD score (26.5% for MELD < 30, 42.9% for MELD between 30 and 40, 75% for MELD > 40; $P = 0.007$) (Table 2). By multivariate analysis, parameters such as CPT score stage C (HR=3.3 95% CI [1.7-6.2]) ($P < 0.001$); serum creatine > 1.5 mg/dL (HR = 2.5 95% CI [1.4-4.3]) ($P = 0.001$) and MELD score over 21 (HR = 2.9 95% CI [1.63-5.21]) ($P < 0.001$) have appeared to significantly influence mortality (Table 2). The overall survival at 3, 6 and 12 mo was 85.4% (95% CI: 78.5-90.1), 76.1% (95% CI: 67.7-82.6) and 67% (95% CI: 57.3-75), respectively. The median and the mean survival time were 142.6 and 318 + 45.1 d, respectively. When the CPT score was considered, survival at 1 year was just 52.1% for CPT score stage C as compared to CPT stage A-B which was over 85% (Figure 1). A MELD score over 21 was synonymous of 50% of survival at 1 year as compared to a MELD score less than 21 for which survival was about 80% (Figure 2). A cut off point of 10, corresponding to the very least value of the CPT score stage C, has been found optimal to predict sensitivity (74%), specificity (49.2%), positive predictive value (38.8%) and negative predictive value (78.4%). A cut off point of 21 for the MELD score has been found optimal to predict sensitivity (48.2%), specificity (69.5%), positive predictive value (41.9%) and negative predictive value (74.6%) (Table 3). However, when the performance of the two scores was

Table 4 Comparison between The Area Under the Curve (AUC) of the CPT score and the MELD score in predicting survival at 3, 6 mo

Survival	Pronostic score	AUC (95% CI)	*P*
At 3 mo	CPT	0.72 (0.64-0.80)	0.68
	MELD	0.75 (0.62-0.88)	
At 6 mo	CPT	0.64 (0.54-0.74)	0.67
	MELD	0.62 (0.49-0.74)	
At 12 mo	CPT	0.69 (0.60-0.78)	0.38
	MELD	0.64 (0.53-0.75)	

CPT: Child-Pugh-Turcott; MELD: Model for End-stage liver disease.

compared at 3, 6 and 12 mo, no significant difference could be found between them. Moreover, the correlation coefficient between the two scores was 0.57 ($P < 0.001$) (Table 4 and Figure 3).

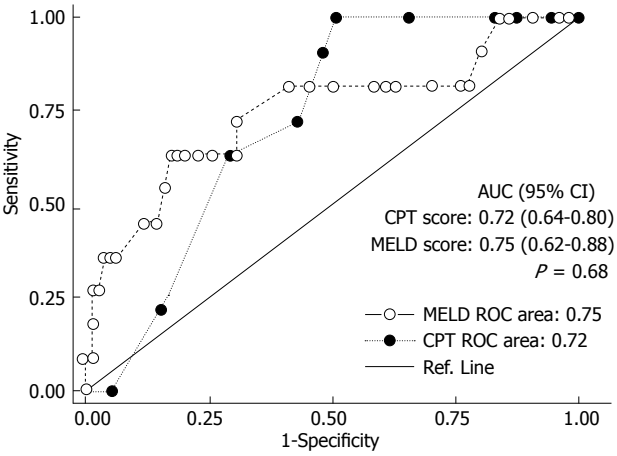


Figure 3 Comparison between AUROC of the CPT score and of the MELD score in predicting survival at 3 mo. CPT: Child-Pugh-Turcott; MELD: Model for End-stage liver disease; Ref. Line: Reference line.

DISCUSSION

About 60% of our patients have displayed a CPT score stage C that amounts to severe liver disease. Hepatitis B has been the main cause of cirrhosis in our study population. The main factors that have appeared to influence mortality were a CPT score stage C (with an optimal cutoff point of 10), renal failure that was present in 1/4 of the patients, and a MELD score over 21. The performances of the two scores were comparable.

Renal failure occurring in the setting of cirrhosis has widely been reported to represent an independent risk factor for mortality[2-6]. However, the CPT[1] score that has been used so far to appreciate prognosis in patients with cirrhosis does not take into account the renal function. This has led, therefore, to the elaboration of the MELD score which formula encompasses serum creatine for the evaluation of survival in patients with cirrhosis[11]. The performance of the two scores has been compared in several studies with conflicting results: some studies[11,18,22,25-27,29] have showed superiority of the MELD score over the CPT score in predicting survival in patients on the transplantation waiting list or in patients awaiting Transjugular Intrahepatic Portosystemic Shunt (TIPS), or even in patients with acute alcoholic hepatitis, while others have found the two score to be comparable[13,14,18,20,21]. Indeed, in the study by Zhang *et al*[27], the area under the curve (AUC) was significantly more with the MELD score than the CPT score: 0.95, 0.85 and 0.83 for the MELD and 0.70, 0.66 and 0.61 for the CPT score ($P < 0.05$) at 3, 6 and 12 mo, respectively. In the study by Papatheodoridis *et al*[28] analysing a cohort of 102 decompensated cirrhotics, the AUC as determined by the MELD score was comparable to that of the CPT score in predicting survival at 3, 6, 12 mo: 0.79, 0.77, 0.78 and 0.79 for the MELD score and 0.73, 0.71, 0.68 and 0.70 for the CPT score. And a recent review by Cholongitas *et al*[31] has highlighted the lack of a clear cut superiority of the MELD score over the CPT score in predicting mortality of cirrhotic patients before and after liver transplantation.

The present study has showed that the two scores were also comparable as the AUC was: 0.75, 0.62, and 0.64 for the MELD score and 0.72, 0.64, 0.69 for the CPT score at 3, 6, and 12 mo, respectively. Moreover, the through value of the MELD score in our study confirms the fact that this value varies from one study to another as it was at 14, 17 and 18 in the Angermayr[24], Cestron[32] and Ferral[25] study, respectively. This could be attributed to the fact that most of our patients were admitted a very late stage of the disease, explaining an elevated mean and median MELD score. The retrospective nature of our study has led to the exclusion of many patients for missing data. Should these patients have been recruited, different findings as the one reported might have been found. However, the present findings could easily be extrapolated to any of our patients since the epidemiological profil of the 172 recruited patients represent the one currently encountered in our daily practice. Our study, which represents the only of its kind to be performed in black African patients with cirrhosis, appears to be of a great interest. Indeed, our population characteristics are quite different from what are currently reported in western countries since our patients are much younger with a diagnosis of cirrhosis made at a very late stage of the disease and hepatitis B being the main cause of cirrhosis. Moreover, the high prevalence of hepatitis B virus in an endemic country, like the Ivory Coast, is often responsible for contamination at birth and in infancy, leading to chronic hepatitis in 30%-50% (when contamination take place in infancy) to 90% (when contamination take place at birth) of the case[34-40]. In addition, the prognosis of our patients is further aggravated by the fact that most of them are diagnosed as having cirrhosis at a very late stage of the disease for different reasons; lack of insurance coverage, delay in hospital visit because of financial problems or cultural considerations. Consequently, few interventions could be offered to these patients as liver transplantation or endoscopic and/or radiological treatments are not currently available in our country. Inversely, Hepatitis C virus and alcohol remain the main causes of cirrhosis in western countries[40-42]. Contamination by hepatitis C virus occurs in adult age and chronic hepatitis related to alcohol represents a disease of adult patient.

Patients with cirrhosis are diagnosed at a very late

stage of the disease. Consequently, mortality is elevated. Renal and liver failures represent independent risk factors of mortality. In the present study, the performance of the MELD score has appeared comparable to that of the CPT score which remains the primary tool to assess patients in the daily practice. The improvement in the MELD score predicting survival ability over the CPT score may necessitate the incorporation in its determination of several others factors such as age, and population characteristics to allow the universal utilizability of this equation. The present study has indeed suggested the impact of the population characteristics on the MELD score level. The use of the CPT score in association with serum creatine as proposed by Angermayr[24] could also be currently performed in daily practice in order to also improve its predicting survival ability.

COMMENTS

Background

Renal failure occurring in the setting of cirrhosis is an independent risk factor of mortality. The Child-Pugh-Turcotte (CPT) score has usually been used to assess the prognosis of patients with cirrhosis. Formulas devoted to include the renal function in the determination of the CPT score have not gained wide acceptance. The Model for End-Stage Liver Disease (MELD) score which encompasses renal function and other factors known to influence mortality in cirrhotic patients has also been found adequate to predict mortality in patients awaiting liver transplantation. The MELD score has thus far been used in the USA for liver graft allocation instead of the CPT score.

Research frontiers

The superiority of one score over the other is subjected to debate as studies comparing the two scores have yielded conflicting results.

Innovations and breakthroughs

Studies performed so far were mostly in western populations, with few in Asian. The present study represents the first of its kind in black Africans. In Africa the main cause of cirrhosis is hepatitis B virus, inversely to hepatitis C and alcohol in western countries. Affecting patients are much younger. Cirrhosis is diagnosed at a very late stage and very little can be done to prolong life of the patients, explaining very high mortality related to it. No difference has been found between the performance of the two scores. However, the optimal cut-off point of the MELD score that is determined from our study appears much higher than any of what that has been published to date.

Applications

The improvement in the MELD score predicting survival ability may necessitate the incorporation in its formulation of several other factors including population characteristics to allow the universal applicability of this equation. Consequently, the setting up of an international collaborative study appears urgent than ever.

Terminology

Cirrhosis: End-stage liver disease. Renal failure: Kidney dysfunction amounting to serum creatine value more than 1.5 mg/dL. CPT score: Five parameters are used to calculate the CPT score. These are hepatic encephalopathy, ascites, and prothrombin in percentage, serum albumin in g/L and bilirubin in mg/L. Each parameter is affected a number going from 1 to 3 depending on its degree of impairment, so that the very least score amounts to 3 and the very most to 15. MELD score: The MELD score is determined through the following equation: [9.57 × Loge creatine (mg/dL) + 3.78 × Loge bilirubin (mg/dL) + 11.20 × Loge INR + 6.43]. Its value appears to vary from to one study to another, depending probably to the population characteristics. Optimal cut-off point: The value of the equation that yields the best sensitivity, specificity, positive predictive value and the negative predictive value.

Peer review

The authors conducted a retrospective study to compare the ability of the CPT and MELD scores to predict mortality in a cohort of black patients with cirrhosis. The key findings of the study are that the CPT and MELD scores had similar predictive ability. Based on the fact that the CPT is easier to compute than the MELD score, the authors recommend the CPT over the MELD score in blacks with cirrhosis. Although not entirely novel, the study does provide interesting and important findings.

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RAPID COMMUNICATION

Expression of pituitary homeobox 1 gene in human gastric carcinogenesis and its clinicopathological significance

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Abstract

AIM: To investigate the effect of pituitary homeobox 1 (PITX1) expression in cases of human gastric cancer on cancer differentiation and progression, and carcinogenesis.

METHODS: Using polyclonal PITX1 antibodies, we studied the expression of PITX1 in normal gastric mucosa, atypical hyperplasia, intestinal metaplasia, and cancer tissue samples from 83 gastric cancer patients by immunohistochemistry. Moreover, semi-reverse transcription polymerase chain reaction (semi-RT-PCR) was performed to detect the mRNA level of PITX1 in three gastric cancer cell lines and a normal gastric epithelial cell line. Subsequently, somatic mutations of the PITX1 gene in 71 gastric cancer patients were analyzed by a combination of denaturing high performance liquid chromatography (DHPLC) and DNA sequencing.

RESULTS: Immunohistochemistry showed that PITX1 was strongly or moderately expressed in the parietal cells of normal gastric mucosa (100%), while 55 (66.3%) out of 83 samples of gastric cancers showed decreased PITX1 expression. Moreover, PITX1 expression was reduced in 20 out of 28 cases (71.5%) of intestinal metaplasia, but in only 1 out of 9 cases (11%) of atypical hyperplasia. More importantly, PITX1 expression was significantly associated with the differentiation, position and invasion depth of gastric cancers (r = -0.316, P < 0.01; r = 0.213, P < 0.05; r = -0.259, P < 0.05, respectively). Similarly, levels of PITX1 mRNA were significantly decreased in 2 gastric cancer cell lines, BGC-823 and SGC-7901, compared with the normal gastric epithelial cell line GES-1 (0.306 ± 0.060 *vs* 0.722 ± 0.102, P < 0.05; 0.356 ± 0.081 *vs* 0.722 ± 0.102, P < 0.05, respectively). Nevertheless, no somatic mutation of PITX1 gene was found in 71 samples of gastric cancer by DHPLC analysis followed by sequencing.

CONCLUSION: Down-regulation of PITX1 may be a frequent molecular event in gastric carcinogenesis. Aberrant levels of PITX1 expression may be closely correlated with the progression and differentiation of gastric cancer.

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Key words: Pituitary homeobox 1; Gastric cancer; Immunohistochemistry; Reverse transcription polymerase chain reaction; Denaturing high performance liquid chromatography

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INTRODUCTION

Cancer develops as a result of multiple genetic and epigenetic alterations[1,2]. Functional imbalances between oncogenes and tumor suppressor genes have been identified in most cancers, including gastric cancer. Better knowledge of the changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment and prevention[3]. Recently, PITX1, first identified as a *bicoid*-related transcription factor involved in proopiomelanocortin (POMC) gene expression[4], was reported to be a tumor suppressor in many parenchyma tumors.

The evidence that *PITX1* gene is a significant tumor suppressor gene in human cancer remains largely circumstantial, but is clearly worth further study. As little is known about the tumor-inhibitory roles of PITX1 in gastric cancer, we focused on the expression of PITX1 in gastric cancer tissues and gastric cancer cell lines; the

potential roles of PITX1 in cancer differentiation and carcinogenesis of the stomach will be discussed.

MATERIALS AND METHODS

Tissue materials

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 83 patients with gastric cancer. The average age of patients was 61 ± 1.5 years and the male/female ratio was 63/20. Histological classification of gastric cancers was performed according to the general rules established by the Japanese Gastric Cancer Association and staging was performed according to the TNM classification of Malignant Tumors (5th edition) in 1997[5].

Tissues from 71 cases of gastric cancer were used for DNA mutation analysis. Tissue fragments were frozen and stored at -80℃ for DNA extraction. DNA was isolated using a standard proteinase K digestion and phenol-choloroform extraction procedure.

All of the above tissues were obtained from the patients who underwent surgery for gastric cancer at the First Affiliated Hospital of China Medical University between December 2001 and November 2004.

Cell lines and cell culture

Three human gastric cancer cell lines, including the poorly-differentiated MGC-803 and BGC-823 lines and the well-differentiated SGC-7901 line, as well as the normal gastric epithelial cell line GES-1, were cultured by the Research Center for Medical Genomics of China Medical University. Among the three gastric cell lines, SGC-7901 was originally obtained from metastatic lymph nodes of gastric cancer while the other two were obtained from gastric primary loci. All cell lines were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum at 37℃, in 99% humidity under 5% CO_2, subcultured every 3 d, and checked routinely to avoid microbial contamination. Those cells growing logarithmically were harvested for total RNA isolation and RT-PCR.

Immunohistochemistry

An S-P Kit (Zhongshan Golden Bridge Biotech Co., Ltd) was used for immunohistochemical analysis. In brief, paraffin sections were deparaffinized in xylene and gradually rehydrated in alcohol. Antigen retrieval was performed by microwave treatment in 0.01 mol/L sodium citrate buffer (pH = 6.0) for 15 min, cooled to room temperature for 1 h, and then washed with phosphate-buffered saline (PBS). After endogenous peroxidase activity was blocked by incubation with 3% methanol for 10 min and washed with PBS, sections were incubated with normal rabbit serum for 1 h to block non-specific staining. Subsequently, sections were treated with polyclonal PITX1 antibodies (Santa Cruz, CA, USA) diluted to 1:300 with PBS, overnight at 4℃ in a humidified chamber. Sections then were incubated with biotinylated anti-rabbit IgG for 10 min and then with peroxidase-labeled streptavidin for 10 min. Staining was completed with a 10-min incubation with 3,3'-diaminobenzidine. Sections were counterstained with 0.1% haematoxylin. Intracytoplasmic and/or intranuclear buffy granular staining in gastric mucous cells was regarded as positive. Ten visual fields were randomly selected from each section, and at least 100 cells were counted in each field.

Omission of primary antibodies acted as a negative control, while normal gastric tissue was regarded as a positive control. Immunohistochemical results were assessed as 'negative' if fewer than 50% of cancer cells were stained. When at least 50% of cancer cells were stained, immunostaining was considered to be 'positive'.

Semi-RT-PCR

Total RNA was isolated from the cell lines using Trizol (Invitrogen, CA, USA), and 1 μg of total RNA was converted to cDNA using a First Strand cDNA Synthesis Kit (Takara, Japan). Then, PCR reactions were performed in volumes of 20 μL. A 1 μL aliquot of the cDNA was used for PCR amplification with the primers 5'-ATGGA CGCCTTCAAGGGGGGC-3' (forward) and 5'-TGCA ACTGCTGGCTTGTGAAG-3' (reverse), resulting in a DNA product of 305 bp. PCR conditions were 95℃ for 1 min, 55℃ for 30 s and 72℃ for 45 s, for 30 cycles. Under the same PCR conditions, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) transcripts (227 bp) were amplified as an external control for RT-PCR analysis from the same cDNA samples, using the following primer pair: 5'-GACC ACAGTCCATGCCATCAC-3' (forward) and 5'-GTCCAC CACCCTGTTGCTGTA-3' (reverse).

PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Electrophoresis strips were analyzed by Fluor-S software. By calculating the ratio of the IDVs (integrated density value) for PITX1 transcripts to those for *GAPDH* transcripts, we compared the PITX1 mRNA levels between gastric cancer cell lines and the normal gastric epithelial cell line.

DHPLC analysis and DNA sequencing

DNA was amplified in a final reaction volume of 20 μL using 100 ng of genomic DNA, 10 × buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 0.27 μmol/L (100 ng) primers and 1.25 units of rTaq polymerase (Takara, Japan).

In order to form the presumed heteroduplex, unpurified PCR products were pretreated by heating at 94℃ followed by cooling down to 25℃ at a rate of 0.03℃/s. DHPLC was carried out using the Transgenomic WAVEMAKE DNA fragment analysis system (Transgenomic, Ohama, USA). PCR products showing aberrant chromatograms on DHPLC were subjected to direct DNA sequencing.

Statistical analysis

The software SSPS for Windows version 11.5 was used for statistical analysis. Correlations between clinicopathological parameters and PITX1 expression were analyzed by χ^2-test, and comparisons of the mRNA levels in different cell lines were performed using *t*-tests. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Expression of PITX1 protein in human gastric cancer samples

As shown in Table 1, all parietal cells (100%) of normal

Table 1 Comparison of the expression of PITX1 in normal tissue and tumor tissue from patients with gastric cancer

	PITX1		
	n	Negative (%)	Positive (%)
Normal tissue	83	0 (0)	100 (100)
Tumor tissue	83	55 (66.3)[b]	28 (33.7)[b]

[b]$P < 0.001$ *vs* normal tissue.

gastric mucosa showed strong or moderate PITX1 staining, while 55 (66.3%) out of 83 gastric cancer samples showed decreased staining for PITX1 ($P < 0.001$). In addition, the expression of PITX1 in intestinal metaplasia tissue samples was reduced in 20 out of 28 cases (71.5%), but in only 1 out of 9 (11%) cases of atypical hyperplasia.

The correlations between clinicopathologic parameters and PITX1 expression in 83 patients are shown in Table 2. PITX1 expression did not differ significantly by age, gender, size, clinical staging and lymph node metastasis. However, significant differences in PITX1 expression were found in tumors with different degrees of differentiation, position or depth of invasion. That is, reduced expression of PITX1 was significantly linked with tumors with poor differentiation, located in the upper and middle parts of the stomach, or with T3/T4 invasion depth ($P < 0.01$, $P < 0.05$, and $P < 0.05$ respectively). Representative results of PITX1 expression in gastric cancer samples are depicted in Figure 1.

Table 2 Relationship between the expression of PITX1 in gastric cancer and biological behavior

	PITX1		
	n	Negative (%)	Positive(%)
Gender			
Male	53	42 (66.7)	21 (33.3)
Female	20	13 (65.0)	7 (35.0)
Age (yr)			
< 60	32	22 (68.7)	10 (31.3)
⩾ 60	51	33 (64.7)	7 (35.3)
Position			
Upper (cardia & fundus)	16	12 (75)	4 (25)
Middle (body)	15	14 (93.3)	1 (6.7)
Lower (antrum)	53	30 (56.6)[a]	23 (43.4)[a]
Depth of invasion			
T1/T2	29	15 (48.4)	16 (51.6)
T3/T4	54	40 (74.1)[c]	14 (25.9)[c]
Clinical stage			
Early stage	17	8 (47.1)	9 (52.9)
Advanced stage	66	47 (71.2)	19 (28.8)
Differentiation type			
Well[1]	38	19 (50)	19 (50)
Poorly[2]	45	36 (80)[b]	9 (20)[b]
Lymph-node metastasis			
Yes	47	33 (70.2)	14 (29.8)
No	36	22 (61.1)	14 (38.9)

[a]$P < 0.05$ *vs* upper and middle part of stomach; [c]$P < 0.05$ *vs* gastric cancer in T1/T2; [b]$P < 0.01$ *vs* well differentiation type. [1]Well-differentiated tumors, for example, papillary adenocarcinoma and tubular adenocarcinoma. [2]Poorly-differentiated tumors, including undifferentiated tumors, signet-cell carcinoma and mucous adenocarcinoma.

Expression analysis of PITX1 mRNA in gastric cancer cell lines and human gastric cancer

RT-PCR was repeated 12 times to analyze the differences in PITX1 mRNA expression between the three gastric cancer cell lines and the normal GES-1 cell line (Figure 2). The average IDV ratios of the gastric cancer cell lines BGC-823 and SGC-7901 were significantly lower than that in the normal gastric cell line GES-1 ($P < 0.05$); there was no statistically significant decrease in IDV ratio in the MGC-803 cell line (Table 3).

PITX1 gene mutation analysis

All of the DHPLC chromatographies in coding area from 71 tumor tissues showed a normal single peak shape except that the deletion of a repeated sequence "gggcgc" in 3' UTR in one sample was found through DHPLC screening and sequencing.

DISCUSSION

Carcinogenesis refers to changes of multiple genes, and is closely related to the corresponding transcription factors (TFs) and their mutual regulation. As a family of TFs, homeobox genes not only play important roles in embryonic development and differentiation, but also control the differentiation and proliferation of mature tissues[6,7]. Class I human homeobox-containing genes (*HOX* genes) have been reported to act as a network of transcriptional regulators involved in the process of cell to cell communication during normal morphogenesis, the alteration of which may contribute to the evolution of cancer[8]. Moreover, a recent study concluded that *HOX* genes may play a critical role in the genesis and progression of gastric cancer[9].

PITX1 and PITX2 are members of the PITX (pituitary homeobox) family, a family of homeobox genes; they have been widely researched and contain a highly conserved homeodomain (HD) with a lysine residue at amino acid position 50, which is responsible for recognition of the TAA (T/G) CC motif in the promoter regions of several target genes[10]. Besides sharing similar DNA binding specificities, PITX1 and PITX2 are co-expressed in the stomodeum and have overlapping expression patterns in stomodeal derivatives[11-13]. Specifically, PITX1 is responsible for the morphology of muscles, tendons and the bones of hind limbs, and is involved in the development of anterior structures as well as pituitary and craniofacial morphogenesis[14,15].

Although PITX1 has essential roles in human development, the possible involvement of PITX1 in human cancer has been suspected since an elegant study by Gidekel *et al*[16] showed that a homeobox gene normally required for the differentiation of a particular tissue can play a causal role in the carcinogenesis of the same tissue if expressed at the wrong time, at the wrong levels, or in the wrong contexts. For instance, PITX1 was found in adrenocorticotropic hormone (ACTH or corticotrophin) -positive tumors as well as in ACTH-negative carcinoids[17]. Expression of PITX1 mRNA might be a potential biomarker of malignancy in patients with

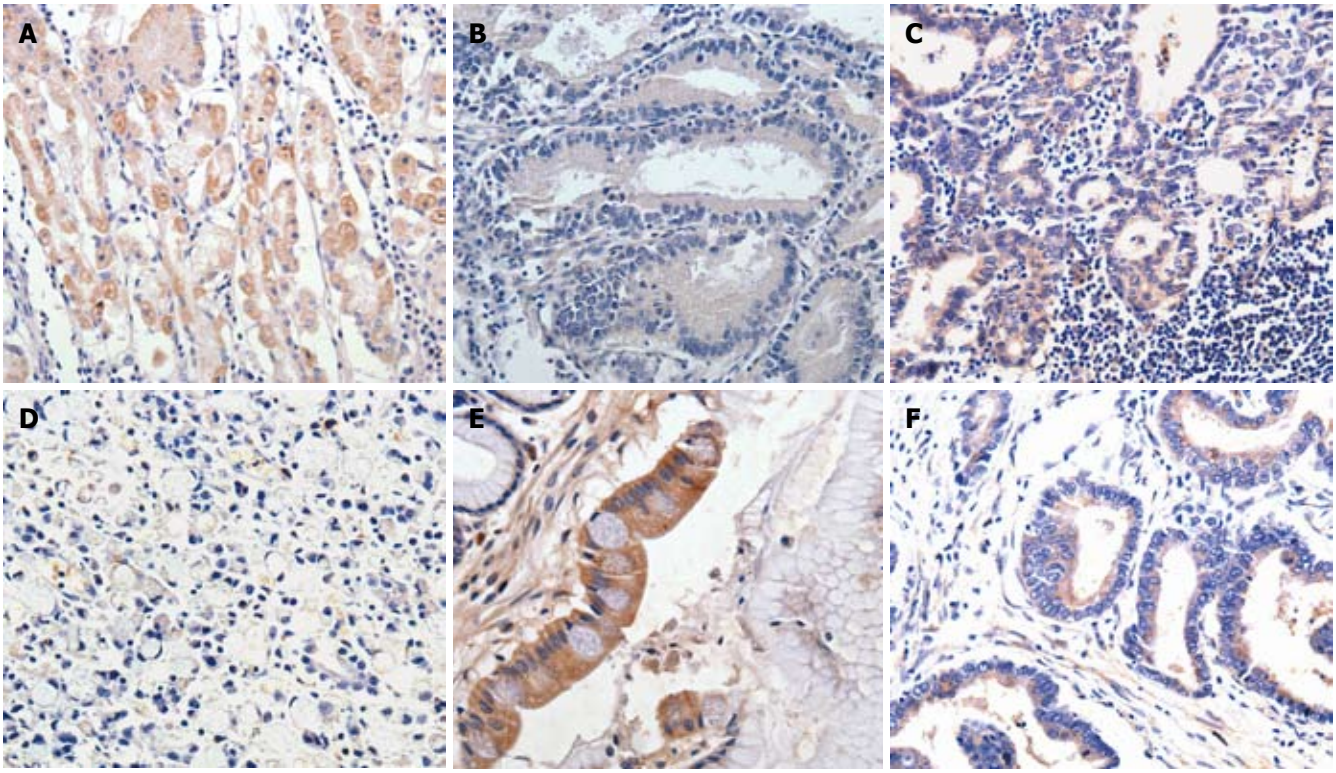


Figure 1 PITX1 expression in normal gastric mucosa and gastric cancer tissue samples (x 400). **A**: Normal gastric mucosa, PITX1 positive; **B**: Well-differentiated gastric carcinoma, PITX1 positive; **C**: Gastric carcinoma with lymph node metastasis, PITX1 positive; **D**: Signet-ring-cell carcinoma (poor-differentiated), PITX1 negative; **E**: Intestinal metaplasia, PITX1 negative; **F**: Atypical hyperplasia, PITX1 positive.

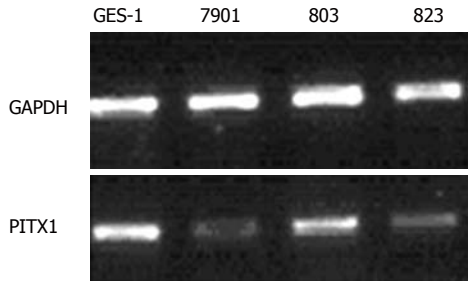


Figure 2 PITX1 expression in three cancer cell lines (SGC-7901, MGC-803 and BGC-823) and the normal gastric epithelial cell line GES-1 (GAPHD serves as the external control).

Table 3 Relationship between the expression of PITX1 in gastric cancer cell lines and the normal gastric cell line

	IDV
MGC-803	0.636 ± 0.092
BGC-823	0.306 ± 0.060[a]
SGC-7901	0.356 ± 0.081[a]
GES- 1	0.722 ± 0.102

[a]$P < 0.05$ *vs* GES-1 cell line.

Barrett's esophagus[18]. Moreover, PITX1 expression is also decreased in lung, prostate and bladder tumor tissues compared with normal control tissues[19-22].

Considering that the expression of the homeobox genes, such as *BARX1* and *PITX1*, is confined to the stomach mesenchyme during gastrointestinal development[23], we studied PITX1 expression in human gastric cancer tissues. In our study, PITX1 expression declined in 63.3% cases of gastric cancer, compared with adjacent normal tissues ($P < 0.001$). Together with former reports on the role of PITX1 in various cancers, our results hinted the negative regulation of PITX1 during carcinogenesis and the development of gastric cancer.

In addition, we found that PITX1 mRNA expression in gastric cancer cell lines was in accordance with its protein expression in immunohistochemistrical results, which agrees with the research of Chen *et al*[19] in lung cancer. Given the insufficient evidence of gastric cancer cell lines, a further study on PITX1 mRNA expression in gastric cancer tissues is necessary to confirm the results of immunohistochemistry and RT-PCR in cell lines.

The low expression of PITX1 suggested that *PITX1* may have a tumor suppressive function, inspiring us to examine its potential role in carcinogenesis. Kolfschoten *et al*[22], reported that *PITX1* regulates the RAS (rat sarcoma) signaling pathway and, therefore, tumorigenesis. As a transcriptional activator, PITX1 might activate RASAL1 to inhibit RAS activity, because PITX1 consensus binding sites, TAA(T/G)CC, were found in the 2 kb upstream promoter region of *RASAL1*, a member of the RAS-GAP family of genes (GTP-activating factors-negative regulators of RAS). Another novel explanation for *PITX1* tumorigenesis came from a study on the role of PITX1 in the transcription of the p53 tumor suppressor gene in human mammary carcinoma (MCF-7) cells, implying that p53 is a direct transcriptional target of PITX1[24].

It has been long recognized that the inactivation of tumor suppressor genes results from mutation, loss of heterozygosity (LOH), aberrant methylation and haploinsufficiency[25,26], among which aberrant promoter hypermethylation is well-known to participate in homeobox gene silencing[27,28]; however, PITX1 promoter hypermethylation has not been found in studies of lung cancer[19]. Therefore, we examined the *PITX1* gene for the presence of mutations in order to explore the mechanism of inactivation of this candidate tumor suppressor gene. We failed to find any mutation in the coding area of *PITX1*, although we did identify the deletion of a repeated sequence in 3'UTR of only one gastric cancer sample. Hence, the absence of detectable mutations or epigenetic silencing suggest that haploinsufficiency is behind the loss of *PITX1* expression[29]. Indeed, it was recently shown that the striking phenotype observed in patients with Rieger's syndrome resulted from decreased PITX2 expression due to deletion mutations in one allele of the gene[30]. As a close relative of PITX2, is PITX1 also subject to haploinsufficiency in gastric carcinogenesis? Further studies are required to investigate the mechanism of PITX1 down-regulation in gastric cancers.

Our study showed that the levels of PITX1 protein are decreased in gastric cancer tissues and that PITX1 mRNA levels are decreased in gastric cancer cell lines, suggesting that *PITX1* is a candidate tumor suppressor gene in gastric cancers, and that its expression is related to the differentiation, position and depth of invasion of gastric cancers.

Knowledge of transcription factors is crucial for understanding the molecular basis of neoplasia. PITX1, a pituitary transcription factor, mediates a plethora of embryonic functions, and its down-regulation in parenchyma tumors may be associated with carcinogenesis. Our study is the first time to explore the link between PITX1 and gastric cancer. Down-regulation of PITX1 both in gastric cancer and gastric cancer cell lines may be involved in the development of gastric cancer and may be a frequent molecular event in gastric carcinogenesis; thus, PITX1 represents a candidate tumor suppressor gene. Therefore, further study should investigate potential mechanisms for modulating PITX1 expression during carcinogenesis and the progression of gastric cancer.

COMMENTS

Background

Gastric cancer is one of the most common types of cancer worldwide. A more complete molecular genetic understanding of gastric cancer will allow the development of specific therapies. Recently, studies on tumor suppressor genes have been highly valued in gastric cancer research owing to their widespread involvement in diverse cellular activities.

Research frontiers

A family of homeobox genes, the PITX family, contains a highly-conserved homeodomain responsible for recognition of the TAA(T/G)CC motif in the promoter regions of several target genes. By virtue of its DNA-binding specificity, PITX1, an important member of this family, has been reported to be involved in many human cancers, such as Barrett's esophagus and lung cancer. The carcinogenic mechanism of PITX1 has been explored in terms of regulation of the RAS signaling pathway and direct activation of p53 transcription.

Innovations and breakthroughs

The study observed a change in the expression levels of PITX1 that parallels the progression of gastric carcinogenesis; we discussed the possible carcinogenic mechanisms used by PITX1 and its clinicopathological significance in gastric cancer. This is the first study to explore the link between PITX1 and gastric cancer.

Applications

The present study suggests that down-regulation of PITX1 may be a frequent molecular event in gastric carcinogenesis. However, the exact function and mechanism of PITX1 in gastric cancer remain to be elucidated.

Terminology

Homeobox genes contain a 180-base-pair segment (the homeobox) that encodes a protein domain (homeodomain) that is involved in binding to and, thus, regulating the expression of DNA; members of the Ras family of small G proteins have been implicated as key intermediates mediating signals from upstream tyrosine kinases to downstream cascades of serine/threonine kinases, which then activate the nuclear factors that control gene expression and protein synthesis.

Peer review

An excellent paper - well written, nicely researched. The results support the hypothesis but further research across multiple centers is needed to establish a positive correlation.

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RAPID COMMUNICATION

Detection of hMSH2 and hMLH1 mutations in Chinese hereditary non-polyposis colorectal cancer kindreds

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Abstract

AIM: To establish and validate the mutation testing for identification and characterization of hereditary non-polyposis colorectal cancer (HNPCC) in suspected Chinese patients.

METHODS: Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. Genomic DNA was extracted after informed consent was obtained. The coding region of hMSH2 and hMLH1 genes was detected by polymerase chain reaction (PCR) and denaturing high-performance liquid chromatography (DHPLC). Mutations identified in the proband by DHPLC were directly sequenced using a 377 DNA sequencer, analyzed with a basic local alignment tool (BLAST), and tested in the corresponding family members by direct DNA sequencing.

RESULTS: Mutations were identified in two Chinese HNPCC kindreds. One was the missense mutation of hMSH2 c.1808A→G resulting in Asp 603 Gly identified in the proband of the fifth HNPCC (HNPCC5) kindred. In the HNP5 kindred, three family members were found to have this mutation and two of them had colorectal cancer. The other mutation of hMLH1 c.1882A→G was identified in the HNP2 kindred's proband, which might be the nonsense mutation analyzed by BLAST.

CONCLUSION: Pedigree investigation and mutation testing of hMSH2 and hMLH1 are the practical methods to identify high-risk HNPCC patients in China.

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INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) syndrome is characterized by autosome-dominantly inherited predisposition to early colorectal carcinoma and extracolonic epithelia-derived tumors most often located in the gastrointestinal and urogenital tracts[1]. The mean age of HNPCC and sporadic colorectal cancer (CRC) patients at diagnosis is 42 years and 65 years, respectively. HNPCC, accounting for approximately 5%-15% of all CRCs, is categorized as Lynch I or Lynch Ⅱ syndrome (according to revised Amsterdam criteria)[2]. Germline mutations of the mismatch repair (MMR) genes identified in HNPCC kindreds, including *MSH2*, *MLH1*, *MSH6*, *PMS1* (promotion of mutS homology 1), *PMS2*, and *MLH3*, have been proved as the major cause for HNPCC by linkage analysis. Mutations in two of these MMR genes, *MSH2* and *MLH1*, account for the majority of the kindreds with HNPCC[3]. Thus, pedigree investigation and MMR gene testing, as the basis for efficient prevention and treatment of HNPCC, are most often used in early diagnosis of at-risk family members and in confirmation of the diagnosis of HNPCC.

CRC is the third life-threatening cancer in China. However, HNPCC pedigree and its predisposition gene have not been extensively studied. We have established a CRC database since 1994 and the follow-up rate is above 90%. We have recently paid attention to hereditary colorectal cancer in South China and found that about 3% of CRC patients have multiple CRCs to which the young are vulnerable[4,5]. Thus, we collected those CRC families and finally obtained eleven independent Chinese kindreds with HNPCC by deep pedigree investigation until January,

2004. Five of them fulfilled the classical Amsterdam criteria. To identify high-risk populations with HNPCC, we tested hMSH2 and hMLH1 mutations in these classical kindreds.

MATERIALS AND METHODS

Patients

The clinical diagnosis of classical HNPCC was established and verified at the Department of Gastrointestinopan creatic surgery, the First Affiliated Hospital of the Sun Yat-Sen University[2]. Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. The study was approved by the Ethical Committee of Sun Yat-Sen University.

DNA isolation

Peripheral blood samples were collected from both patients and their family members in each Chinese kindred after informed consent was obtained for genetic analysis. DNA was extracted directly from leukocytes following the standard procedures.

All exons of *MLH1* and *MSH2* were amplified for mutation testing. Samples used were amplified in 20 mL reaction volume containing approximately 100 ng DNA in 50 mmol/L KCl, 10 mmol/L Tris-HCl, 1.5 mmol/L $MgCl_2$, 200 mmol/L each dNTP, 0.01% gelatin, 1 U Taq-polymerase, and 20-40 pmol each primer. The PCR amplification conditions were as follows: denaturation at 95℃ for 5 min, followed by 30 cycles of denaturation at 95℃ for 30 s, annealing at fragment-specific annealing temperature for 1 min, extension at 72℃ for 1 min, and a final extension at 72℃ for 7 min. The primers, annealing temperature, and size of the PCR products for each of the investigated hMLH1 and hMSH2 exons are described elsewhere[6-8]. The primers were synthesized by Shanghai Bio-Chemical Corporation.

DHPLC analysis

The amplified PCR fragments were screened for sequence variants by denaturing high pressure liquid chromatography (DHPLC) on a WAVE DNA fragment analysis system (Transgenomic, San Jose, CA, USA). The running conditions for each amplicon (available upon request) were determined by the Wavemaker 3.4.4 software (Transgenomic, San Jose, CA, USA) based on the DNA sequence. Five mL of each PCR product (containing 50-100 ng of DNA) was denaturated at 95℃ for 3 min and then gradually reannealed by decreasing the sample temperature from 95℃ to 65℃ over 30 min. PCR products were then separated at a flow rate of 0.9 mL/min with a linear acetonitrile gradient. Generally, analysis took approximately 10 min including column regeneration and re-equilibration to starting conditions. The column mobile phase consisted of a mixture of 0.1 mol/L triethylamine acetate (pH 7.0) with (buffer B) or without (buffer A) 25% acetonitrile. Samples displaying variant elution peaks in each run were chosen for sequence analysis. For some amplicons displaying variations in elution profiles of control samples, all samples were sequenced.

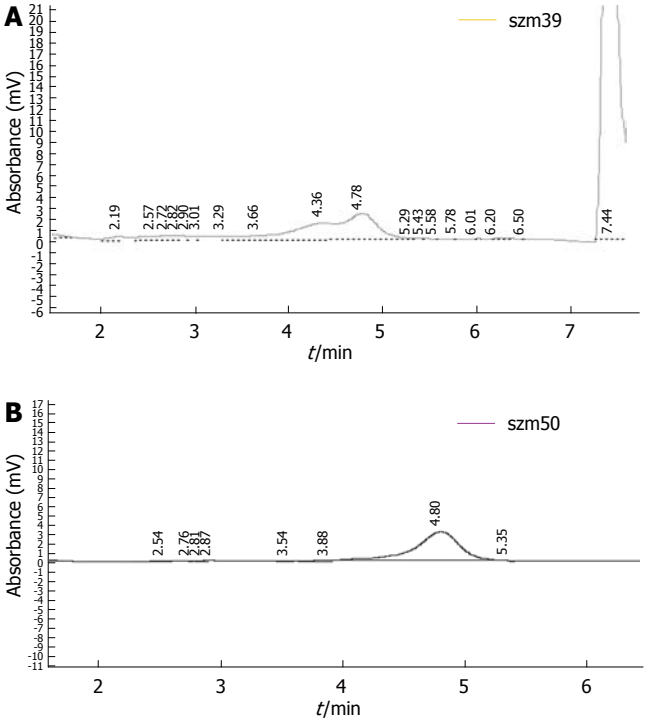


Figure 1 DHPLC showing variant elution peaks (**A**) and normal elution peaks (**B**) in exon 12 of hMSH2 gene.

DNA sequencing

DHPLC variants were confirmed by direct sequencing of independently amplified PCR products of the amplicons, in both sense and antisense direction, using the same primers. Sequencing was performed with Big Dye™ terminator cycle sequencing ready reaction kit (Applied Biosystems, Inc., Foster City, CA) on an ABI Prism™ 377 DNA sequencer following the standard conditions recommended by the manufacturer. Sequences obtained were aligned and compared to published wild-type sequences by Sequencher 3.1.1 analysis software.

Mutations identified in the kindred's proband were tested among their family members by direct DNA sequencing.

RESULTS

Among the 5 probands of the five Chinese HNPCC kindreds, 180 PCR products were screened by DHPLC. Two PCR products found with variant elution peaks by DHPLC were identified to have mutations. One was hMSH2 c.1808A→G resulting in Asp 603 Gly identified in the HNP5 kindred's proband and tested among the family members (Figures 1-3). The other was hMLH1 c.1882A →G identified in the HNP2 kindred's proband (Figures 4 and 5), which was not tested among the family members, because it might be the nonsense mutation analyzed by BLAST.

As shown in Figure 3, in the HNPCC5 kindred, there were four colorectal carcinoma patients in two successive generations, and three of these were diagnosed before the age of 45 years. The proband developed endometrial carcinoma at the age of 61 years, bladder carcinoma at the age of 66 years and colorectal carcinoma at the age of 72

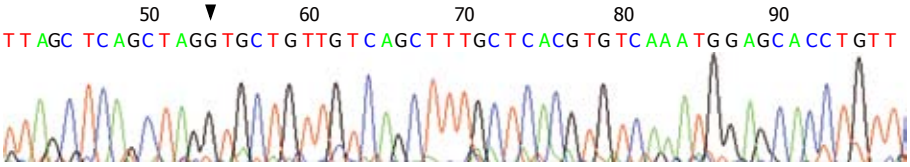


Figure 2 HPLC variants confirmed by direct sequencing displaying a single nucleotide substitution of c.1808A→G in exon 12 of hMSH2 gene.

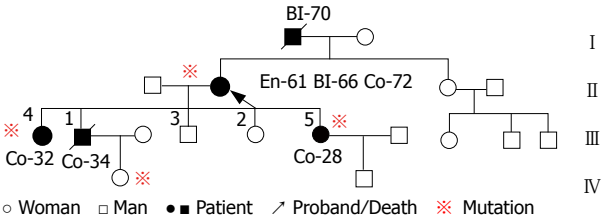


Figure 3 Pedigree tree of HNPCC 5 kindred. Co: Colon; Bl: Bladder; En: Endometrium.

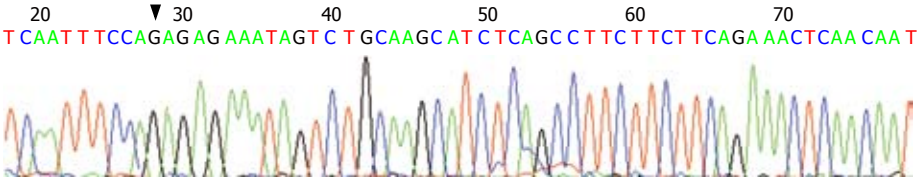


Figure 4 DHPLC variants confirmed by direct sequencing revealing a single nucleotide substitution of c.1882A→G in exon 16 of hMLH1 gene.

years, while his father developed bladder carcinoma at the age of 70 years. In addition, patient Ⅲ-1 had colorectal carcinoma at the age of 34 years and died of synchronous hepatic metastasis.

In the HNP5 kindred, each PCR product from nine proband's family members was tested by direct DNA sequencing. Only three of them were found to have the missense mutation in hMSH2 at position A1808G. The missense mutation sequence variant was found in exon 12 of hMSH2 gene. It was a single nucleotide substitution of c.1808A→G (Figure 2), which resulted in Asp 603 Gly of hMSH2 (NCBI Ref. Seq. NM 000251 and NP 000242 for mRNA and protein, respectively). Proband and patient Ⅲ-4 and -5 had this mutation and developed colorectal carcinoma. Patient Ⅲ-1's daughter had no colorectal disease even though she had this mutation, and was still under follow-up. No mutation was found in the others.

DISCUSSION

CRC is one of the most common cancers and its clinical selection criteria for cancer families were first established in Amsterdam in 1990 by the International Collaborative Group on HNPCC and modified in 1999[2]. Detection of constitutional mutations in genes associated with predisposition to cancer is practical in molecular diagnosis. Data suggest that molecular testing is much more efficient, if analyses are focused on a limited number of alterations[9-13]. Detection of germline mutation carriers is an efficient method to define high-risk CRC patients. Identification of germline mutations of either hMSH2 or hMLH1 could be performed in 50%-70% of families meeting the Amsterdam criteria for HNPCC, whereas the families not complying with these criteria show a much lower frequency of the MMR gene mutations[14-17]. Thus, hMSH2 or hMLH1 gene testing is most often used in early diagnosis of at-risk family members with HNPCC.

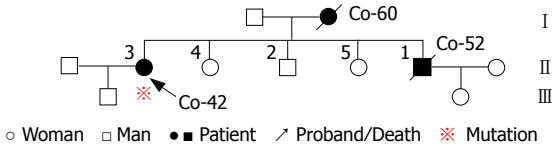


Figure 5 Pedigree tree of HNPCC 2 kindred. Co: Colon.

To identify a population-specific panel of mutations, it is crucial to describe them in all ethnic groups.

Even though CRC is common in China, few studies on HNPCC pedigree and its predisposition gene are available[4,17]. In our study, HNPCC kindreds were collected and hMSH2 and hMLH1 gene mutations were tested in order to find high-risk CRC populations. Two mutations were found among the five Chinese HNPCC kindreds. In the 5HNPCC kindreds, missense mutation was found in four members, three of them developed colorectal carcinoma. Although it has not been confirmed as a germline mutation yet, it may be an important factor for CRC development. Thus, the patient Ⅲ-1's daughter with this mutation should be closely followed up.

Approximately 20% of patients with colorectal cancer have a genetic component and HNPCC is the most common autosome dominant hereditary syndrome predisposing to colorectal cancer[18,19]. Various methods have been described to screen for HNPCC and directly test for mismatch repair gene mutations[20-27]. For patients with available tumor specimens, MSI and IHC are widely performed. Ruszkiewicz *et al*[28] reported that immunohistochemistry is an alternative method for assessment of MSI status, which is fast and relatively inexpensive compared with MSI testing. Some reports[26,29,30] suggest that detection of MSI and IHC for hMSH2/hMLH1 proteins is a reliable pre-screening test for hMLH1/hMSH2 germline mutations in families suspected of having HNPCC. Because China is a developing country with a large population and the incidence rate of CRC

increases, a screening protocol specific for the Chinese population is necessary to detect the high-risk populations with HNPCC. hMSH2 and hMLH1 gene mutation testing is a practical method for detecting HNPCC in Chinese population. Meanwhile, further research should be performed in Chinese HNPCC kindreds with germline mutations.

COMMENTS

Background

Approximately 20% of colorectal cancer patients have a genetic component and hereditary non-polyposis colorectal cancer (HNPCC) is the most common autosome dominant hereditary syndrome predisposing to colorectal cancer. Various methods have been described to screen for HNPCC and directly test for mismatch repair gene mutations. Even though CRC is common in China, few studies on HNPCC pedigree and its predisposition gene are available.

Research frontiers

Data suggest that molecular testing is much more efficient when analyses are focused on a limited number of alterations. Detection of germline mutation carriers is an efficient method to define high-risk CRC patients. Germline mutations of either hMSH2 or hMLH1 can be identified in 50%-70% of families meeting the Amsterdam criteria for HNPCC. Thus, hMSH2 or hMLH1 gene testing is most often used in early diagnosis of at-risk family members with HNPCC.

Innovations and breakthroughs

Five independent Chinese kindreds with HNPCC fulfilling the classical Amsterdam criteria were collected. Mutations were identified in two Chinese HNPCC kindreds. One was the missense mutation of hMSH2 c.1808A→G resulting in Asp 603 Gly, the other was the mutation of hMLH1 c.1882A→G .

Applications

Molecular pathological tests should be performed in order to identify individuals and at-risk family members with HNPCC. Close follow-up and intensive surveillance should be performed.

Terminology

Denaturing high-performance liquid chromatography (DHPLC) is a method for separating DNA duplexes that differ in the identity of one or more base pairs. The method is believed to be most efficient at the site of mutation. Basic local alignment search tool (BLAST) is powerful to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be obtained from well designed queries and alignments. BLAST 2.0 can rapidly search for nucleotide and protein in their databases.

Peer review

The results of this clinical study indicate that mutation testing for hMSH2 and hMLH1 by PCR and direct sequencing is a preferable method to identify high-risk HNPCC patients.

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RAPID COMMUNICATION

Assessment of duodenal circular drainage in treatment of superior mesenteric artery syndrome

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Abstract

AIM: To assess the clinical value of duodenal circular drainage for superior mesenteric artery syndrome (SMAS).

METHODS: Forty-seven cases of SMAS were treated with duodenal circular drainage from 1959 to 2001. Clinical data were analyzed retrospectively.

RESULTS: In this group, good effects were achieved in 39 cases treated with duodenal circular drainage after 2-15 years of follow-up. The other eight cases were first treated with anterior repositioning of the duodenum (two cases), duodenojejunostomy (five cases), subtotal gastrectomy and billroth Ⅱ gastrojejunostomy (one case), but vomiting was not relieved until duodenal circular drainage was performed again. A follow-up study of 8-10 years revealed satisfactory results in these eight patients.

CONCLUSION: In SMAS, if the reversed peristalsis is strong and continuous, and vomiting occurs frequently, the symptom can not be relieved even if the obstruction of duodenum is removed surgically. The key treatment is the relief of reversed peristalsis. The duodenal circular drainage can resolve the drainage direction of duodenal content, thus relieving the symptom of vomiting.

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Key words: Duodenum; Superior mesenteric artery syndrome; Circular drainage operation

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INTRODUCTION

The pathogenesis of superior mesenteric artery syndrome (SAMS) is so complicated[1-3] that there are different operations for treating SAMS[4-7], such as ligament of Treitz amputation, gastrojejunostomy, subtotal gastrectomy and Billroth Ⅱ gastrojejunostomy, duodenojejunostomy, anterior reposition of the duodenum, duodenal circular drainage operation, *etc*[8-10]. The last one of all these procedures is suitable for the patients who have failed in other operations or have stronger and continuous reversed peristalsis[11]. Among a total of 108 patients with SAMS who received operations, 47 patents were treated with duodenal circular drainage from 1959 to 2001 in the Second Affiliated Hospital of Harbin Medical University.

MATERIALS AND METHODS

Patients

This study included 28 men and 19 women, aged from 16 to 50 years (average 29), including 38 patients aged 16-35 years. The clinical course of the diseases lasted 2-11 years, averaging 3.5 years.

Clinical characteristics

The onset of SAMS was slow in all 47 patients. The symptoms were upper abdomen expanding, dull and bursting pain. The pain may present in upper abdomen, upper left or right of umbilicus and right hypochondrium, which was not relieved after body position change. The vomiting could be triggered by eating and drinking. Body position change (knee-chest position) did not make any difference. The vomit contained bill-stained material and no dejection smell. Six patients were misdiagnosed as having obstruction of pylorus (five patients were misdiagnosed by other hospitals) and 14 patients had ejecting vomiting. Three patients were misdiagnosed as having gastric cancer because of severe dehydration and disorder of electrolyte induced by a high frequency of vomiting.

All patients suffered from severe dehydration, disorder of electrolyte and loss of weight. The patients weighed from 45-53 kg in 28 men and 40-42 kg in 19 women. The patients' upper abdomen was distended and lower abdomen was plainness. There were no abdominal muscle guarding and obviously tenderness, but shaping of the stomach and succussion splash occasionally. There was no change of borhorygmus.

Laboratory examination

Obvious expansion in stomach, and descending and



Figure 1 Barium X ray study in duodenal circular drainage operation.

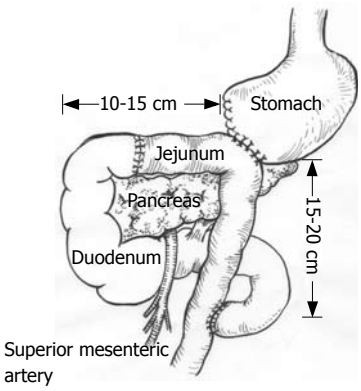


Figure 2 Duodenal circular drainage operation.

transverse portion of duodenum were found in all the patients in barium X-ray study. The barium was delayed in the ascending portion of duodenum which looked like pen trace (Figure 1). The reversed peristalsis of duodenum was much stronger than direct peristalsis. It looked like the movement of pendulum, and barium could reverse to stomach. If the patients were put in prone position, a smaller portion of barium could go through the obstructive site and enter into jejunum. Five patients had histories of peptic ulcer at the same time. Duodenum expanded obviously, reaching 7.5 cm-8 cm in diameter, in 26 patients with SMAS.

Duodenum air charge test in operation

A total of 108 SMAS patients received duodenum air charge test in operation. After interdiction duodenum with an elastic tourniquet, about 200-400 mL air was injected into the proximal duodenum. The diameter of duodenum expanded to 5.5 cm-6.0 cm was considered as light expansion, 6.5 cm-7.0 cm as moderate expansion, and 8 cm or broader as severe expansion. In our group, the duodenum expanded to 8 cm in 47 patients.

Operation

All the 47 patients received duodenal circular drainage operation finally. Two patients received anterior reposition of the duodenum and gastrojejunostomy and five patients underwent duodenojejunostomy before receiving this operation. Vomiting was not alleviated after these procedures in these patients, and they received duodenal circular drainage operation 3-6 mo after the first surgery. One patient suffered from duodenal partial obstruction after subtotal gastrectomy and Billroth Ⅱ gastrojejunostomy, which induced obstructive jaundice and suppurative cholangitis because the bilious and pancreatic liquid can not be drained easily. The reason was that when duodenal nub was closed, a bowel segment was formed between duodenal nub and the position compressed by the duodenum. The patient was cured after duodenal circular drainage operation two mo after the first operation.

Operational methods

Under epidural anesthesia or general anesthesia with tracheal intubation, midline incisions were made in upper abdomen in all patients. We performed duodenum air charge test first. If the duodenum was distended and widened obviously to 8 cm or wider, distal stomach should be resected, and if the patients had gastric ulcer, subtotal gastrectomy should be performed. Afterwards the jejunum should be cut off 10 cm-15 cm from ligament of Treitz (sometimes 20 cm according to blood vessel supply). Distal jejunum was drawn through mesentery of transverse colon and was retrocolically anastomosed end to end with duodenal cap. Half length stomach (about 5 cm) was anastomosed to jejunum end to side 10 cm-15 cm from the jejunum-duodenal cap anastomosis. Finally proximal jejunum was anastomosed with distal jejunum end to side 15 cm-20 cm below the gastrojejunoanastomosed stoma. The incision was stitched after washing of abdominal cavity (Figure 2).

RESULTS

All 47 received gastroenteric X-ray test (60-100 mL of 76% meglumine diatrizoate injection or 200 mL barium was taken orally). Contrast agent could pass the gastroenteroanastomosed stoma and jejunal-jejunostosed stoma successfully. No stricture of anastomotic stoma was found in our group. The contrast agent was refluxed to the end to end duodenal cap anastomosis in 6 patients. Meglumine diatrizoate injection (76%) was administered in 2 cases after 10 min. Barium was influxed to the jejunum, and some was refluxed to the end to end duodenal cap anastomosis, and partly refluxed to stomach. Six patients had bile refluxed gastritis 20-25 d after operation. The symptom disappeared after one month treatment with pectous bismuth and domperidone. The contrast agent was administered to the jejunum and no reflux was found in barium X-ray study in other patients. Reverse peristalsis disappeared in all patients finally.

All patients were released from symptoms in our group 10-15 d after operation. All patients had a good status in a follow-up study of 2-15 years. The ratio of follow-up was 100%, including 47 cases followed within two years. No abdominal expanding, pain and vomiting occurred. The weight increased by 8 kg in 25 patients, 5 kg in 15 patients and 7 kg in 3 patients. Gastroenteric barium X-ray studies showed no stricture in two anastomosed stomas and no contrast agent reflux was observed in these cases (Figure 3). Thirty-eight (80.9%) patients were followed in the third year and no abdominal pain or expanding and vomiting were found and appetite all became normal, with weights



Figure 3 Barium X ray study after duodenal circular drainage operation.

of 60-64 kg in male patients and 46-51 kg in female. In 21 patients who received ultrasonic examination, no obvious expansion was found in duodenum, the diameter of duodenum being 3 cm-5 cm. Eighteen (38.3%) patients have been followed up to the present. Eight patients had received other operations before the duodenal circular drainage operation. A follow-up study of 8-10 years revealed good effects in these patients. No abdominal pain, expansion, and vomiting were found in all the 18 patients and all with normal appetite. Gastroenteric barium X-ray study showed no stricture and ulcer in two anastomosed stomas and no contrast agent reflux was observed in these cases. The weight was 60-64 kg in male patients and 45-51 kg in female. No obvious expansion was found in duodenum in the 18 patients, the diameter of duodenum being 4-5 cm[12-14].

DISCUSSION

Theoretic basis and indications for duodenal circular drainage operation

The movements of duodenum are complicated and the most important movements are reversed peristalsis and direct peristalsis[15-18]. The direct peristalsis is greater than reversed peristalsis under normal circumstances. Complete or partial obstruction could be induced when the ascending portion of duodenum was compressed by superior mesenteric artery[19-22]. The reversed peristalsis is greater than the direct peristalsis, which is the most important reason inducing opening of pyloric duct[23,24]. The patients may suffer from frequent vomiting in such situation[25-27]. If reversed peristalsis exists continually and chronically, the reversed peristalsis will be hardly eliminated. Releasing from obstruction by operation can not alleviate clinical symptoms because the strongly reversed peristalsis still exists. Therefore, emphasis should laid on eliminating the reversed peristalsis to alleviate frequent vomiting. Duodenojejunostomy is commonly used but can not stop the vomiting while duodenal circular drainage operation can manage the drainage direction of duodenal contents. When duodenum takes peristalsis directly, the duodenal contents could only partly enter into jejunum through anastomosis of proximal and distal jejunum. When the strongly reversed peristalsis with the duodenal contents could come into jejunum completely through anastomosis of distal jejunum and duodenal cap, the vomiting is eliminated completely[28-30].

Indications for duodenal circular drainage are as follows: (a) the history of the disease is up to 2 years, especially 5 years; (b) frequent vomiting, no obvious abdominal pain but marked abdominal distention after eating. The pain may be alleviated after vomiting; (c) the vomiting can not be alleviated by changing body position (prostrate position after eating), it may result from chronic reversed peristalsis; (d) the descending and transverse portion of duodenum is markedly expanded and show strong and frequent reversed peristalsis in barium X-ray test, pyloric duct is opened, the stomach is expanded and atonic; (e) the cavity of duodenum was expanded to 8 cm or more in air charge test, and the pressure of duodenum is 15-17 cm H_2O; and (f) the patients are not cured after duodenojejunostomy and anterior reposition of the duodenum. Duodenal circular drainage should conform to the indications above. If the patients do not meet with these indications, duodenal circular drainage can induce disorder of duodenal function.

Assessment of duodenal circular drainage

Duodenal circular drainage can eliminate frequent vomiting induced by chronic reversed peristalsis in SMAS. The ulcer focus and most of gastric body can be resected (gastrojejunostomy similar to Billroth Ⅱ) if the patients suffer from the ulcer intercurrently. The operation also can eliminate blind end between anastomosis and obstructive site after duodenojejunostomy and can not induce blind loop syndrome. No ulcer or stricture of anastomosis occurred in our group.

There are still several disadvantages of duodenal circular drainage operation. The operation is complicated as compared with duodenojejunostomy, and the patients may suffer from more damages as a result of the operation; small stomach syndrome and anemia can be induced by resection of most gastric body; the mucous membrane of alimentary tract can not be nourished by gastrin after resection of gastric antrum; and bilious reflux gastritis may occur after resection of sphincter of pylorus. A few patients had bilious reflux gastritis in our group and recovered within one month.

COMMENTS

Background

Superior mesenteric artery syndrome (SMAS) is an uncommon disease which can result in postprandial epigastric pain, nausea, vomiting, anorexia and weight loss. The pathogenesis of SMAS is ascribed to compression of the third part of the duodenum in the angle between the aorta and the superior mesenteric artery. If conservative management fails, surgical options should be applied.

Research frontiers

Conservative therapy with nutritional supplementation is the initial treatment, and surgery is reserved for those who do not respond to medical therapy. The main character of the SMAS is that the reversed peristalsis is greater than the direct peristalsis, which is the most important reason inducing the symptoms. If reversed peristalsis exists continually and chronically, the reversed peristalsis will be hardly eliminated. Only releasing from obstruction by operation can not alleviate clinical symptoms because the strong reversed peristalsis still exists. Therefore, emphasis should be laid on eliminating reversed peristalsis to alleviate frequent vomiting.

Related publications

Surgical options include open or laparoscopic duodenojejunostomy or ligament of Treitz amputation, gastrojejunostomy, subtotal gastrectomy and Billroth Ⅱ gastrojejunostomy, duodenojejunostomy, anterior reposition of the duodenum, duodenal circular drainage operation, and so on. SMAS is associated with a wide range of predisposing conditions and surgical procedures, more studies are needed to better define the optimal treatment for this disease.

Innovations and breakthroughs

We and many other authors had performed other operations for SMAS. However, some patients showed bad results, especially those with stronger reversed peristalsis. So we designed the operation of duodenal circular drainage. In practice, the operation achieved good results in most patients, All patients showed good status during a follow-up study of 2-15 years.

Applications

Forty-seven cases of SMAS were treated with duodenal circular drainage operation from 1959 to 2001 and had good effects after 2-15 years of follow-up.

Terminology

Duodenal circular drainage: A kind of operation we designed to treat SMAS. The distal jejunum is retrocolically anastomosed with duodenal cap end to end. Half length stomach is anastomosed to jejunum end to side 10 cm-15 cm from the jejunum-duodenal cap anastomosis. Proximal jejunum was anastomosed with distal jejunum end to side 15 cm-20 cm below the gastrojejunoanastomosed stoma.

Peer review

The manuscript evaluates the clinical value of duodenal circular drainage operation for superior mesenteric artery syndrome. The method is simple and correct. Discussion is well organized and conclusions are valuable.

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RAPID COMMUNICATION

Association between the presence of *H pylori* in the liver and hepatocellular carcinoma: A meta-analysis

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Abstract

AIM: To evaluate the arguments for and against the possible roles of *H pylori* in hepatocellular carcinoma (HCC).

METHODS: We performed a systematic review of all relevant studies published in the literature. A total of 103 clinical trials and reports were identified, but only 10 trials qualified under our selection criteria. A meta-analysis was carried out by a biostatistician according to the Cochrane Reviewers' Handbook recommended by The Cochrane Collaboration.

RESULTS: Nine case-control studies and one retrospective cross sectional study were included in the final analysis. Overall the prevalence of *H pylori* infection was 53.3% (129 of 242) in cases and 10.4% (29 of 280) in controls, and the summary odds ratio for the association of *H pylori* infection with the risk for HCC (using the fixed-effects model, which accounted for the homogeneity across the 10 studies) was determined to be 13.63 (95% CI, 7.90-23.49).

CONCLUSION: Our analysis showed a positive association between *H pylori* infection and the risk of HCC, with an indication of possible publication bias and possible confounders due to study designs that showed results of less pronounced associations.

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Key words: *H pylori*; Hepatocellular carcinoma; Meta-analysis; Publication bias

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INTRODUCTION

The profound impact of hepatocellular carcinoma (HCC) on human health is known worldwide[1]. Persistent hepatitis B virus (HBV) and hepatitis C virus (HCV) infections and aflatoxins are the main causes of HCC[2]. The real risk factors for HCC may be far more numerous than the known causes. The infectious agent *Helicobacter hepaticus* (*H hepaticus*), for example, first described by Ward *et al* in 1994, has recently received new attention for its role in causing chronic active hepatitis and associated liver tumors[3].

Since *H pylori* was first cultivated from a human gastric biopsy specimen in 1982, it has become apparent that *H pylori* infection is correlated with gastric cancer and mucosa-associated lymphoid tissue lymphoma. More recently, researchers have reported that *Helicobacter spp.* have been identified in liver tissue resected from patients with HCC[4]. Experimental infection by *Helicobacter hepaticus* in mice causes chronic hepatitis and HCC. It is highly noteworthy that *H pylori* was found in liver tissues resected from patients with HCC[5-7].

The question of whether *H pylori* could play a role in the development of HCC remains controversial. Many conflicting reports have been published to date; thus, we performed a systematic rev iew of all of the relevant studies published in the literature to evaluate the arguments for and against the possible roles of *H pylori* in HCC.

MATERIALS AND METHODS

Selection criteria

We searched different databases, including the *Cochrane Controlled Trials Register on The Cochrane Library* Issue 1, 2007, MEDLINE (January, 1989-March, 2007), EMBASE.com (January, 1989-March, 2007) and the China Biological Medicine Database (CBMdisc) (January, 1989-March,

Table 1 Characteristics of 10 studies investigating the association between the presence of *H pylori* infection in liver and hepatocellular carcinoma

Reference	Country, year of publication	*H pylori* positivity/total subjects		Type of controls (*n*)	Age range (mean), yr	
		Cases	Controls		Cases	Controls
Pellicano *et al*[6]	Italy, 2004	17/20	2/6	Metastatic cancer (*n* = 6)	NA	NA
Ito *et al*[7]	Japan, 2004	13/15	0/17	Cirrhotic liver tissue specimens (*n* = 10), normal liver tissue specimens (*n* = 7)	36-73 (59.2)	NA
Coppola *et al*[8]	Italy, 2003	0/21	0/34	Metastatic liver carcinoma (*n* = 7), chronic hepatitis (*n* = 27)	NA	NA
Dore *et al*[9]	Italy, 2002	6/11	5/30	Chronic viral hepatitis without (*n* = 18) or with (*n* = 12) cirrhosis	19-78 (54.9)	49-78 (65.2)
Avenaud *et al*[10]	France, 2000	8/8	1/8	Patients without primary liver carcinoma (*n* = 8)	NA	NA
Nilsson *et al*[11]	Sweden, 2001	12/16	0/20	Metastatic liver carcinoma (*n* = 20)	NA	NA
Zhang *et al*[12]	China, 2004	16/48	2/37	Liver cirrhosis (*n* = 12), pericacinomatous tissues (*n* = 10), benign tumor of liver (*n* = 9), chronic hepatitis (*n* = 6)	25-67 (46.5)	35-65 (42.5)
Huang *et al*[13]	China, 2004	16/38	0/30	Liver cirrhosis (*n* = 15), benign tumor of liver (*n* = 15)	NA	NA
Li N *et al*[14]	China, 2006	22/34	0/20	Liver external injury (*n* = 5) giant hemangioma (*n* = 5), macrosis hepatic cyst (*n* = 3), ntrahepatic bile duct stone (*n* = 7)	28-71 (52)	30-68 (48)
Rocha *et al*[15]	France, 2005	19/31	19/78	Non-cirrhotic chronic hepatitis C (*n* = 24), HCV Positive cirrhosis without HCC (*n* = 29), HCV Positive cirrhosis and HCC (*n* = 25)	NA	NA

2007), for the terms 'hepatocellular carcinoma' and '*H pylori*'. The reference lists of pertinent reviews and retrieved articles were also checked for the identification of additional studies. We also performed a full manual search from the bibliographies of selected papers. Studies were identified by two researchers, independently; the two lists were compared and discrepancies were resolved. We also contacted the authors of studies containing relevant information, who did not report the results necessary for this analysis. Unpublished data were also accepted if an abstract was available and further information was obtained from the author.

In the meta-analysis, the following inclusive selection criteria were set and reviewed by two independent investigators: (1) each trial is an independent case-controlled study; (2) the purpose of all studies and statistical methods is similar; (3) the numbers of cases, controls and positive rate of *helicobacter* (the presence of *H pylori* DNA sequences in human liver or other standard methods for definition of *H pylori* infection in the liver) are concrete; (4) the study groups have definite HCC by pathology, histology and operation; (5) only studies that selected HCC patients as cases and subjects with other liver diseases or normal liver tissue specimens as controls are included.

The following exclusive selection criteria were set: (1) incomplete raw data; (2) repetitive reports (if more than one version of the same study was retrieved, only the most recent is used); (3) the positive rate of *helicobacter* is not detected by the presence of *H pylori* DNA sequences or other standard methods for detection of *H pylori* infection in liver (that is, all subjects are tested for the presence in serum of IgG antibodies against *H pylori*).

A total of 103 clinical trials and reports were identified and only 10 trials[6-15] qualified on the basis of our selection criteria. The studies were independently evaluated by two researchers. Discrepancies in the evaluations of some studies were resolved by discussion between the reviewers. The main features of the trials included in the meta-analysis are shown in Table 1.

Data extraction and outcomes

Data extracted included year of publication, country of origin, number of cases and controls, characteristics of controls, age of participants, prevalence of *H pylori* infection in cases and controls, and reported odds ratios (OR). All available studies were reviewed by two investigators independently. Reference 18 is a retrospective cross-sectional study; the others are case-controlled studies.

Statistical analysis

The meta-analysis was carried out by a biostatistician (Chen AJ) according to the Cochrane Reviewers' Handbook recommended by The Cochrane Collaboration. First, a pooled OR was calculated using the fixed-effect model. The heterogeneity of the studies was examined using the DL Q statistic[16]. Because the results were homogeneous ($P = 0.07$), a fixed-effects model was employed using the DerSimonian and Laird (DL) methods. A pooled OR was presented as a standard plot with 95 percent confidence intervals (CI). Begg and Mazumdar's proposed adjusted rank correlation test[17] and Egger's linear regression approach[10] were used to measure publication bias, which was shown as a funnel plot (Figure 1). Fixed (Figure 2A) and random-effects models (Figure 2B) were also used to perform sensitivity-analysis to assess the reliability of meta-analysis. The statistical package RevMan version 5.0 (provided by The Cochrane Collaboration, Oxford, England) was used for statistical analyses.

RESULTS

Nine case-controlled studies and one retrospective cross-sectional study were were identified and reviewed, as shown in Table 1[6-15].

In the meta-analysis, the overall prevalence of *H pylori* infection was 53.3% (129 of 242) in cases and 10.4% (29 of 280) in controls, and a summary OR for the association of *H pylori* infection with the risk for hepatocellular carcinoma (using the fixed-effects model, which accounted for the homogeneity across the 10 studies) was determined to be 13.63 (95% CI, 7.90-23.49) (Figure 2A). Figure 2A shows the ORs and 95% CIs of each study, and the summary OR determined by meta-analysis. The proportion of the total variation in study estimates, because of heterogeneity, was 44.0% (heterogeneity test statistics χ^2 = 14.28, on 8 df, P = 0.07, I^2 = 44.0%). A random-effects model was also used to perform sensitivity-analysis to assess the reliability of meta-analysis, as shown in Figure 2B.

Graphical and statistical evaluation of publication bias

Publication bias was assessed for all pooled ORs with confidence intervals using Begg's test[17,18]. This bias is shown as a funnel plot in Figure 1.

The funnel plot method was used to assess the possible presence of publication bias[19,20]. It consists of plotting each study's OR on a logarithmic scale (horizontal axis) against its SE (vertical axis), with an informal visual examination of the funnel plot graph to check for funnel plot asymmetry as an indication of potential publication bias. Studies of smaller size normally have a wider distribution of results than larger studies, because of a higher degree of random variation, and the ORs scatter more widely at the bottom of such a graph, with the spread narrowing with increasing precision among larger studies. In the absence of a publication bias, the graph appears as a symmetrical inverted funnel, as the risk estimates should be symmetrically distributed around the midpoint value (that is, the summary OR). Publication bias may occur if smaller studies showing no significant results remain unpublished, leading to an asymmetrical appearance of the funnel plot with a gap at the bottom of the graph.

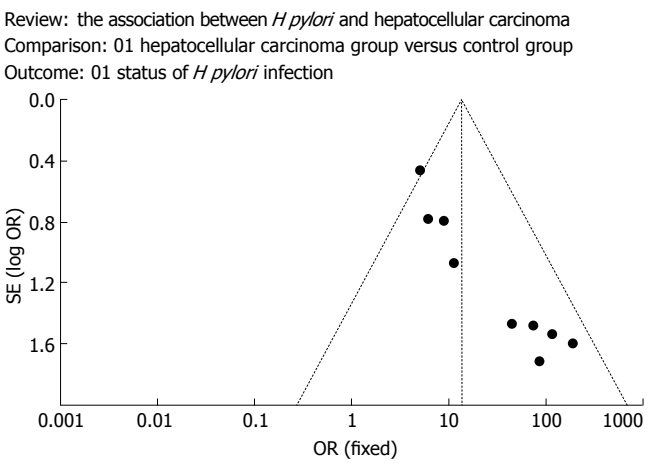


Figure 1 Funnel plot to explore publication bias. The graphical funnel plot of the 10 published studies appears to be asymmetrical.

DISCUSSION

H pylori infection is a classical model with which to study cancer development as a consequence of chronic inflammation. The estimated total of infection-attributed malignancies per year is 1.9 million cases or 17.8% of the global cancer burden[21]. Among the principal carcinogenic agents, *H pylori* is a leading factor, being responsible for 5.5% of all cancers. *H pylori* was classified as a type I carcinogen by the International Agency for Research on Cancer in 1994[22]. A striking finding indicated by Ward *et al* is that bacterial infection of the liver in healthy A/JCr male mice is capable of inducing a strong inflammatory change in the parenchyma (for example, hepatitis) leading to HCC.

We analyzed the published evidence investigating the association between *H pylori* infection and HCC. Studies concerning this possible association have been undertaken since the early 2000s. To our knowledge, this is the first published meta-analysis investigating this association. The summary OR for the association of evidence of *H pylori* infection and the risk for HCC was estimated to be 13.63 with a 95% CI from 7.90 to 23.49, with a confound of study design (lower for studies of prospective design and higher for retrospective case-controlled studies).

In our study, only publications in English or Chinese were used for evaluation. 'Meta-analytical' research on 29 meta-analyses investigating language bias has provided evidence that the OR estimated in meta-analyses from non-English publications are on average 0.8-fold (95% CI, 0.7-1.0) the OR estimates from English-written publications[23]. Therefore, even if we had not searched for non-English publications, this might have introduced only a small bias in the overall findings, which, in our opinion, would not have altered our main conclusions.

Several other points should be considered when interpreting the results of our study.

First, the positive rate of *helicobacter* of the most studies was detected by the presence of *H pylori* DNA sequences. Polymerase chain reaction (PCR) amplification using two sets of primers located in the 16S ribosomal DNA (rDNA) was used to detect the presence of bacteria, but this is not a 'gold standard' method for detecting *H pylori* infection in the liver. Histology with standard stains and culturing maybe more precise than 16S rDNA; however, false negatives are likely. Research in this area has been limited by the lack of a gold standard for the diagnosis of these organisms in the liver. Most published data to date have been based on molecular techniques that detect the DNA of *Helicobacter* species in liver tissues, rather than evidence of viable organisms in the liver.

Secondly, Reference 18 is a retrospective cross-sectional study, whereas the other studies are case-controlled studies. Such studies are generally lower in quality for use as prospective design studies and higher in quality as retrospective case-controlled studies. These observational studies are more prone to bias than randomized clinical trial (RCT) studies.

Third, in this analysis, graphical and statistical methods for testing and adjusting for a possible publication bias and a test for potential heterogeneity between studies were performed. A graphical funnel plot of the 10 published studies was asymmetrical, which may suggest the probable

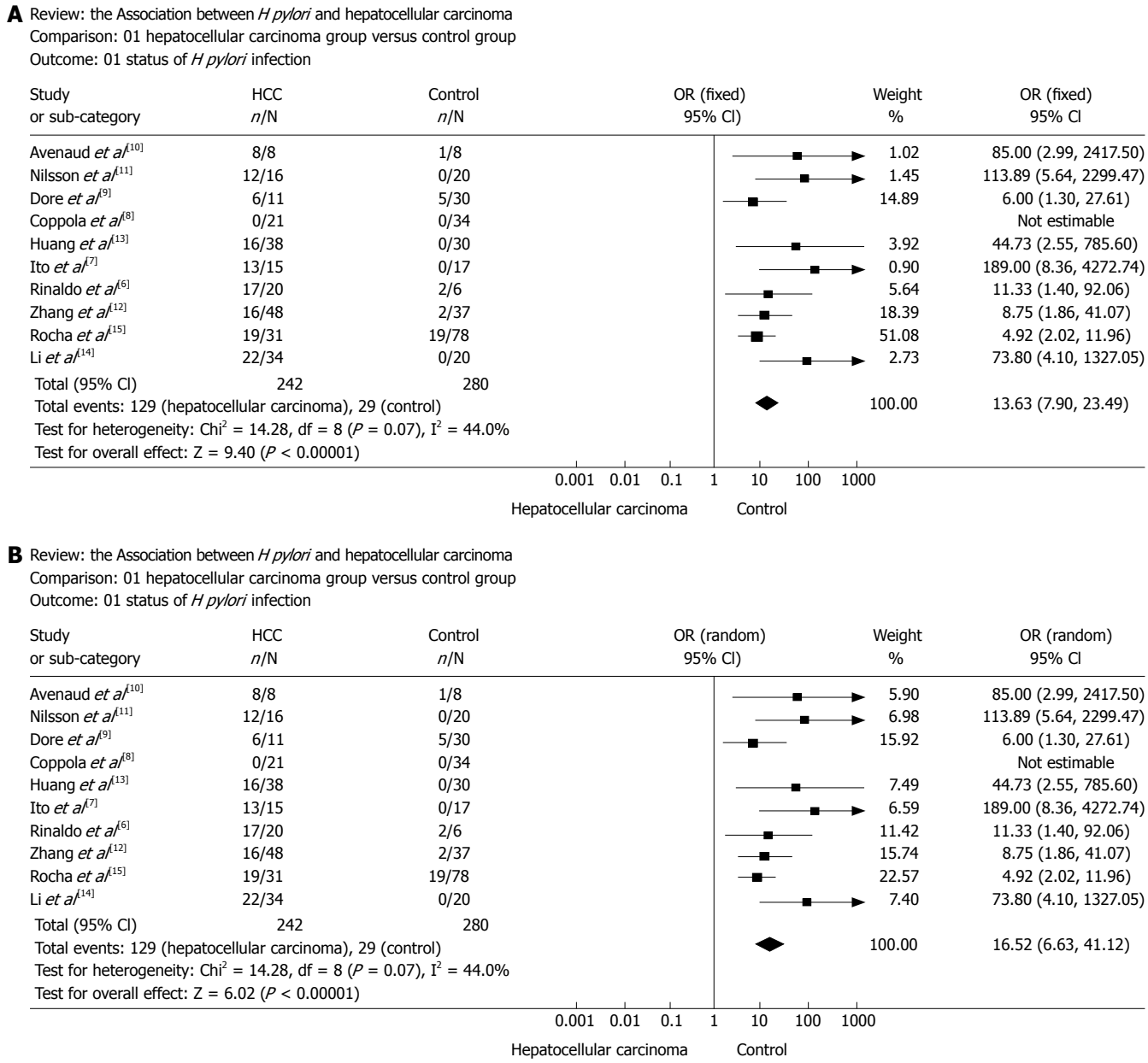


Figure 2 **A**: Forest plot of a meta-analysis of the association between *H pylori* and hepatocellular carcinoma risk (fixed-effects mode). It shows the ORs and 95% CI of each study, and the summary OR determined by meta-analysis. The proportion of total variation in study estimates, because of heterogeneity, was 44.0% (heterogeneity test statistics χ^2 = 14.28, on 8 df, P = 0.07, I^2 = 44.0%); **B**: Sensitivity-analysis: A Forest plot of a meta-analysis of the association between *H pylori* and hepatocellular carcinoma risk (random-effects mode).

existence of publication bias, although this may occur if some studies showing no significant results remained unpublished or if such negative studies are few.

Additionally, because the information used in our research was based on data from observational studies, the characteristics of each study population and the different methodologies of these studies should be taken into account when interpreting the results of our analysis. For example, different inclusion criteria for selection of the participants might have influenced the results of this research. Differences in the age distribution, different countries and different types of control groups (cirrhotic patients, patients with chronic viral hepatitis without or with cirrhosis, patients without primary liver carcinoma, patients with metastatic liver carcinoma, pericacinomatous tissues, benign tumors of the liver, liver external injuries, giant hemangiomas, macrosis hepatic cysts, and intrahepatic bile duct stones) could also be among the potential causes of variation in the studies' estimates.

Most studies did not control for the matching variables in the analysis, and the risk for HCC was not controlled for possible confounders such as HBV or HCV, except in Reference 18.

Only the articles of Dore *et al*[9] and Rocha *et al*[15] demonstrated the association of *Helicobacter* species with hepatitis C cirrhosis and HCC. These two studies included a large series of patients and examined both tumor and cirrhotic liver tissue samples from patients with HCV-positive HCC. *Helicobacter* DNA was found in a small percentage of liver biopsies from controls as well as from patients with chronic hepatitis C (4.2% and 3.5%, respectively). However, the prevalence of *Helicobacter*

species was high in patients with HCV-positive cirrhosis and in those with cirrhosis and HCC (68% and 61%, respectively). In nearly all cancer tissues, *Helicobacter* DNA was detected and identified as *Helicobacter* pullorum or *H pylori*-like organisms. The authors suggested a possible causal role of these bacteria in the progression of chronic hepatitis C and the development of HCC.

Furthermore, although we tried to maximize our efforts to identify all relevant published studies in peer-reviewed journals, it is possible that some escaped our attention.

There is some evidence to suggest that *Helicobacter* infection may be associated with an increased risk of extra-gastric malignancies[24]. The presence of *Helicobacter* species in liver tissues from patients with different liver diseases, including hepatic neoplasias, has been reported by numerous authors. The most intriguing hypothesis is that these bacteria might play a role in the development of HCC[25].

Despite its limitations, the present analysis has some implications: as relatively few studies are available in this field and current evidence remains limited, the necessity to conduct large studies with an adequate methodological quality, properly controlling for possible confounds in order to obtain valid results, should be emphasized; for example, tissue from unaffected liver metastatic carcinoma could be used as a suitable control.

In conclusion, our analysis showed a positive association between *H pylori* infection and the risk of HCC. We obtained from our meta-analysis a summary OR of 13.63 for the association of *H pylori* infection and HCC, with an indication of possible publication bias and confounds of study design, with less pronounced associations in prospective studies than in retrospective studies. Therefore, this risk increase should be interpreted with caution. Better designed and better controlled studies are needed to clarify the strength of this association and the possible causal role of *H pylori* infection in patients with HCC, and further prospective studies are required to prove this hypothesis. Given the importance of this potential association, further verification is warranted.

COMMENTS

Background

The real risk factors for hepatocellular carcinoma (HCC) may be far more than the known causes. A new infectious agent, *Helicobacter hepaticus* (*H hepaticus*), causing chronic active hepatitis and associated liver tumors, has been described by Ward *et al*. The question of whether *H pylori* could play a role in the development of HCC remains controversial. Many conflicting reports have been published to date, so we performed a systematic review of all of the relevant studies published in the literature to evaluate the arguments for and against the possible roles of *H pylori* in HCC.

Research frontiers

Since *H pylori* was first cultivated from human gastric biopsy specimens in 1982, it has become apparent that *H pylori* infection is correlated with gastric cancer and mucosa-associated lymphoid tissue lymphoma. More recently, researchers have reported that *Helicobacter spp*. were identified in liver tissue resected from patients with HCC. Experimental infection by *Helicobacter hepaticus* in mice causes chronic hepatitis and HCC. It is highly noteworthy that *H pylori* was found in liver tissue resected from patients with HCC.

Innovations and breakthroughs

To our knowledge, this is the first published meta-analysis investigating the association between *H pylori* and hepatocellular carcinoma risk.

Applications

Our analysis showed a positive association between *H pylori* infection and the risk of hepatocellular carcinoma, with an indication of possible publication bias and confound of study design, with less pronounced associations in prospective studies than in retrospective studies. Therefore, this risk increase should be interpreted with caution; better designed and better controlled studies are needed to clarify the strength of the association and the possible causal role of *H pylori* infection in hepatocellular carcinoma. Further prospective studies are requested to prove this hypothesis; given the importance of this potential association, further verification is warranted.

Terminology

Meta analysis: In statistics, a meta-analysis combines the result of several studies that address a set of related research hypotheses. *H pylori*: *H pylori* is a bacteria that can cause digestive illnesses, including gastritis and peptic ulcer disease. More recently, researchers have reported that *Helicobacter spp*. were identified in liver tissue resected from patients with hepatocellular carcinoma.

Peer review

Authors evaluated the arguments for and against the possible roles of *H pylori* in HCC through a systematic review of all relevant studies published in the literature. The analysis showed a positive association between *H pylori* infection and the risk of HCC.

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RAPID COMMUNICATION

Small intestinal bacteria overgrowth decreases small intestinal motility in the NASH rats

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Abstract

AIM: To explore the relationship between small intestinal motility and small intestinal bacteria overgrowth (SIBO) in Nonalcoholic steatohepatitis (NASH), and to investigate the effect of SIBO on the pathogenesis of NASH in rats. The effect of cidomycin in alleviating severity of NASH is also studied.

METHODS: Forty eight rats were randomly divided into NASH group (n = 16), cidomycin group (n = 16) and control group (n = 16). Then each group were subdivided into small intestinal motility group (n = 8), bacteria group (n = 8) respectively. A semi-solid colored marker was used for monitoring small intestinal transit. The proximal small intestine was harvested under sterile condition and processed for quantitation for aerobes (*E. coli*) and anaerobes (Lactobacilli). Liver pathologic score was calculated to qualify the severity of hepatitis. Serum ALT, AST levels were detected to evaluate the severity of hepatitis.

RESULTS: Small intestinal transit was inhibited in NASH group (P < 0.01). Rats treated with cidomycin had higher small intestine transit rate than rats in NASH group (P < 0.01). High fat diet resulted in quantitative alterations in the aerobes (*E. coli*) but not in the anoerobics (Lactobacill). There was an increase in the number of *E. coli* in the proximal small intestinal flora in NASH group than in control group (1.70 ± 0.12 log10 (CFU/g) *vs* 1.28 ± 0.07 log10 (CFU/g), P < 0.01). TNF-α concentration was significantly higher in NASH group than in control group (1.13 ± 0.15 mmol/L *vs* 0.57 ± 0.09 mmol/L, P < 0.01). TNF-α concentration was lower in cidomycin group than in NASH group (0.63 ± 0.09 mmol/L *vs* 1.13 ± 0.15 mmol/L, P < 0.01). Treatment with cidomycin showed its effect by significantly lowering serum ALT, AST and TNF-α levels of NASH rats.

CONCLUSION: SIBO may decrease small intestinal movement in NASH rats. SIBO may be an important pathogenesis of Nash. And treatment with cidomycin by mouth can alleviate the severity of NASH.

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Key words: Nonalcoholic steatohepatitis; Small intestinal motility; Small intestinal bacteria overgrowth treatment

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INTRODUCTION

With much more prevalence of nonalcoholic fatty liver disease (NAFLD) than previously thought noticed, there has been an explosion on the research of pathogenesis of NAFLD. NAFLD may develop into cirrhosis of liver through NASH, in some cases, to hepatocellular carcinoma[1-5], Insulin resistance and peroxidative injury were thought to place an important role in the pathogenesis of NASH[6-12]. But there are still other mechanisms that can trigger and maintain NASH[13-18]. Small intestinal bacterial overgrowth has been reported to place a role in the pathogenesis of NASH, endotoxin and TNF-α being the possible mediator[19-21]. But contrary to this hypothesis, in another study, antibiotic treatment did not normalize aminotransferase levels in NASH patients[22]. Therefore more study is needed on the influence of SIBO on the pathogenesis of NASH and the curative effect of antibiotic on NASH. Alejandro S[22] found that prolonged Orocecal Transit Time (OCTT) in NAFLD patients coexist with SIBO. It is generally thought that prolonged OCTT is the cause of SIBO. But it is not clear if alteration of intestinal motility is a cause or a consequence of SIBO.

We established an NASH animal model by high fat diet to explore the relationship between intestinal motility and SIBO in NASH. The relationship between SIBO and NASH, and the curative effect of antibiotic on NASH were also studied.

MATERIALS AND METHODS

Materials

Forty eight male SD strain rats were used in this study.

Rats were housed individually in cages at constant room temperature in a 12-h light/dark cycle and had free access to laboratory feed and water. Semi-solid colored marker (carbon-ink 85%, gum acacia 10%, activated charcoal 5%) was made in our laboratory. Bacteria evaluation kit (API 20) was purchased from French Biomerieux CO. TNF-α kit was purchased from Technology Development Center of the General Hospital of PLA.

Establishment of animal mode

Forty eight SD rats were randomly divided into NASH group, cidomycin group and control group. Each group were subdivided into small intestinal motility group (n = 8) and bacteria group (n = 8) respectively. Rats in NASH group and cidomycin group were fed with high fat diet that was made by ordinary diet (88%) plus fat (10%) and cholesterol (2%) (supported by Qinglongshan Nursery) Rats in control group were fed with ordinary diet (supported by Qinglongshan Nursery). Furthermore, rats in cidomycin group were treated with cidomycin (12Mu ig qd) after eight weeks of high fat diet while those in the other two groups were treated with isotonic Na chloride (1 mL/d ig qd). At the end of the twelfth week, when the rats in NASH group and cidomycin group had received high fat diet for 12 wk, all the rats were killed, the serum levels of aminotransferase, TNF-α were tested and the histology of liver specimen was observed by H&E staining.

Measurement of small intestinal transit

Rats in small intestinal motility group was deprived of food for 24 h and water for 12 h prior to measurement of small intestinal transit. Then 1.0 mL semi-solid colored marker was administered into stomach by orogastric gavage. Twenty minutes later, the rat was killed, abdomen was opened and small intestine was dissected. The distance traveled by the marker was calculated. The small intestinal transit was represented by ratio of the distance traveled by the marker to the total length of the small intestine.

Histological evaluation

The liver pathologic score was calculated according to the lecture[23]: Inflammation in portal canal area was denoted by "P", inflammation inside lobules of liver by "L", piecemeal necrosis by "PN" and bridging necrosis by "BN". According to the severity of inflammation and necrosis, every item was scored from one to four. The total score of the liver inflammation was "P+L+2PN+2BN".

Measurement of small intestinal bacteria

Rats in the bacteria group was deprived of food for 24 h and water for 12 h prior to measurement of small intestinal bacteria. Then the rat was killed, the abdomen was opened and the proximal small intestine was harvested under sterile condition. A two-centimeter-long small intestine was dissected from the point about ten centimeters from pylorus, then rinsed with sterile saline thrice, and next, the leftover was sucked by sterile filter paper. After weighing, the leftover and 2 mL sterile saline were placed in a sterile glass homogenizer and homogenized. The homogenate was diluted with sterile saline at the ratio of 1:1, then, 100 μL dilution was plated on SS agar (*E. coli*) and MRS agar (Lactobacilli) respectively. The quantity of *E. coli* was determined after 24 h of aerobic cultivation at 37℃. While the quantity of Lactobacilli was determined after 48 h of anaerobic cultivation at 37℃. The number of Colony forming units (CFU) of bacteria was quantified. The quality of aseptic manipulation was evaluated by inoculating swab of abdominal cavity on sheep-blood agar. The serum TNF-α levels were detected, using radio-immunity method, in our laboratory.

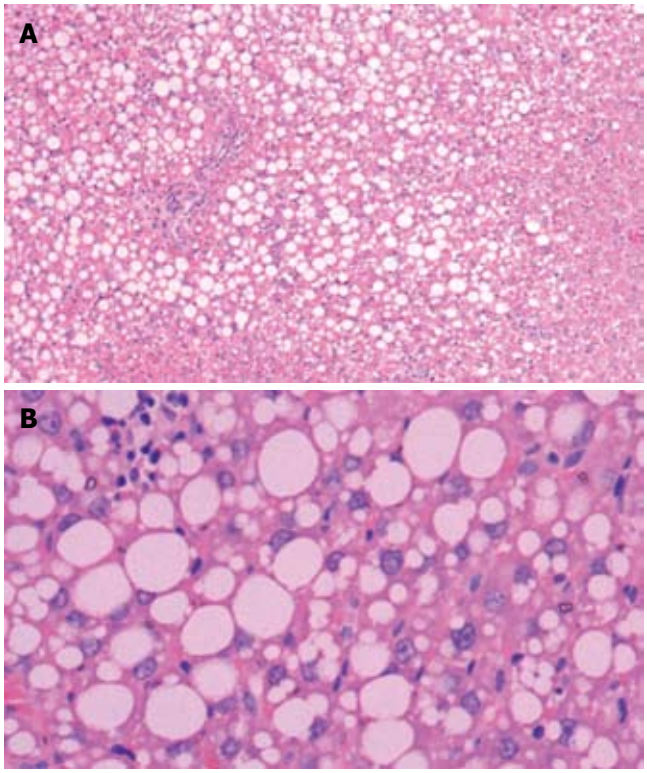


Figure 1 Pathological changes of liver after 12 wk of high fat diet. **A**: Fat drops in more than one thirds of liver cells (HE, × 40); **B**: Lymphocytes in hepatic steatosis background (HE, × 100).

Statistical analysis

Throughout this report, data were expressed as mean ± SD. Experimental results were analyzed by one-factor analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

The weight of the model group rise *vs* controls was 455.38 ± 11.48g *vs* 395.38 ± 10.91 g, $P < 0.05$.

Pathologically a NASH model has been successfully made (Figure 1).

TNF-α, liver pathologic score, serum ALT and AST levels are higher in the NASH group than in the other two groups indicating a NASH model has been successfully made ($P < 0.01$, $P < 0.01$, $P < 0.05$). There are no statistical differences between the control group and the cidomycin group in serum TNF-α level. Liver pathologic score, serum ALT and AST levels are higher in cidomycin group than in control group ($P < 0.01$) (Table 1).

Table 1 The concentration of TNF-α, liver pathologic score, serum ALT and AST levels (mean ± SD)

	n	TNF-α (ng/mL)	ALT (U/L)	AST (U/L)	Liver pathologic score
Control group	8	0.57 ± 0.09[b]	39.73 ± 5.10	168.00 ± 16.41	1.00 ± 0.93
NASH group	8	1.13 ± 0.15	86.63 ± 20.91[d]	379.63 ± 61.73[d]	6.00 ± 1.20[d]
Cidomycin group	8	0.63 ± 0.09[b]	70.88 ± 11.93[a,d]	318.75 ± 52.62[a,d]	4.25 ± 1.58[a,d]

[b]$P < 0.01$ *vs* NASH group; [d]$P < 0.01$ *vs* control group; [a]$P < 0.05$ *vs* NASH group.

Table 2 Small intestinal bacteria, small intestinal transit rate (mean ± SD)

	n	*E. coli* [$\log_{10}$(CFU/g)]	*Lactobacilli* [$\log_{10}$(CFU/g)]	Small intestinal motility(fraction)
Control group	8	1.28 ± 0.07	1.67 ± 0.16	0.58 ± 0.06
NASH group	8	1.70 ± 0.12[b]	1.69 ± 0.16	0.39 ± 0.11[b]
Cidomycin group	8	1.17 ± 0.08[a,d]	1.81 ± 0.13	0.51 ± 0.07[d]

[b]$P < 0.01$ *vs* control group; [a]$P < 0.05$ *vs* control group; [d]$P < 0.01$ *vs* NASH group.

The number of *E. coli* in the proximal small intestinal flora are more in the NASH group than in the control group and cidomycin group ($P < 0.01$, $P < 0.01$). Small intestinal motility are weaker in NASH group than in control group and cidomycin group ($P < 0.01$, $P < 0.01$). The number of *E. coli* are less in cidomycin group than in control group ($P < 0.05$). There are no statistic difference in small intestinal motility between control group and cidomycin group. There are no statistical differences in the number of Lactobacilli between every two groups of the three (Table 2).

DISCUSSION

It was found that NASH coexist with SIBO[19,24]. In our study excessive multiplication of *E. coli* and increased serum level of aminopherase coexist in NASH rats, that was consistent with previous studies. We thought that SIBO is an important, though not the only, pathogenesis of NASH by the fact that antibacterial treatment can alleviate the severity of NASH. We also found the level of aminopherase went up and down with serum level of TNF-α. It strongly supported TNF-α to be an important mediator in the promotion of NASH by SIBO. In our study, rats treated with cidomycin had a serum level of aminopherase slightly higher than controls and a serum level of TNF-α similar to controls. The reason or this phenomenon might be that TNF-α is not the only mediator. Generally endotoxemia was thought to be a link between SIBO and elevated TNF-α level[19,20]. But Wigg A J[25] observed that serum levels of TNF-α and endotoxin were not parallel in NASH patients. Given the disadvantage of clinical trials to control experimental conditions, and the disadvantage of C^{14} breath test in identification of bacteria, animal experiments are necessary to find the link between SIBO and NASH. Our study found a significant increase in the number of *E. coli*, a widely known source of endotoxin, in the proximal small intestinal flora in NASH group. It might support endotoxin to be the promoter to TNF-α in NASH.

With respect to the causality between SIBO and small intestinal motility in NASH, ALEJANDRO-S[22] found that delayed intestinal transit coexist with SIBO in patients effected by NASH. We also observed that NASH rats suffered both delayed intestinal motility and an increase in intestinal E. coli. It is widely believed that delayed intestinal motility could cause SIBO. Leveau P *et al*[26] noticed a delay in intestinal transit time, appeared as an early event in acute pancreatitis, preceding intestinal bacterial overgrowth, and suggested that impairment in intestinal motility probably played a role in the development of bacterial overgrowth. Gangarosa[27] demonstrated that intestinal motility served as a normal cleansing mechanism of the intestine, and drugs that decreased this motility might facilitate replication of pathogens and their attachment to or invasion of the intestinal tissue. Nieuwenhuijs *et al*[28,29], clarified the role of the migrating motor complex (MMC) in the regulation of small intestinal microflora by the fact that the disruption of MMC with morphine promotes duodenal bacterial overgrowth. Since MMC can also coordinate with the movement of the pylorus, gut and cholecyst, the alteration of MMC necessarily has an influence on the secretion of bile (the inhibiter of gram-negative bacilli). MMC might influence intestinal bacteria *via* another mechanism. Grzesiuk *et al*[30], demonstrated that intestinal bacteria, particularly those adhering to intestinal epithelial cells, were exposed to electric fields and currents generated by the muscular activity of the small intestine and that the myoelectrical activity of the duodenum, through action on cell membrane, can affect cell division of intestinal bacteria.

Though it is generally believed that insulin resistance can explain the delay of small intestinal motility in NASH, and abnormality of intestinal bacteria may be caused by delayed intestinal motility, in our experiment, treatment with antibiotics improved the abnormality of small intestinal motility in NASH rats, indicating that small intestinal bacterial can also regulate intestinal motility in NASH rats. The interpretation may be founded on the influence microflora has on intestinal smooth muscle cell myoelectricity action. Huseby *et al*[31] demonstrated that after introduction of conventional intestinal microflora the interval between activity fronts of the MMC in proximal jejunum of germ-free rats was reduced. Sjogren *et al*[32] reported that bacterial adherence to the intestinal mucosa appeared to be important in eliciting the abnormal myoelectric responses. Different intestinal bacterial genus may have different influence on intestinal motility. *E. coli* adherence to intestinal mucosa delayed spike bursts[29], reduced the frequency and amplitude of the intestinal contractions and inhibit small intestinal transit[29]. while

Lactobacillus promoted regular spike burst activity, reduced the MMC period and accelerated small intestinal transit[29]. In our study the overgrowth of *E. coli* coexist with the reduction of small intestinal motility, and the elimination of *E. coli* could increase the small intestinal motility, strongly supporting *E. coli* to be an important mediator in the small intestinal motility in NASH rats. In our study Lactobacillus didn't change according to high fat diet or cidomycin treatment, indicating Lactobacillus may not be important in the pathogenesis of NASH.

The results of this study suggest that SIBO may be not only the result of but also a promoter to delayed intestinal motility in NASH. There might be a vicious circle between SIBO and delayed intestinal motility in NASH rats. SIBO can trigger NASH *via* TNF-α, and treatment with cidomycin orally can alleviate the severity of NASH.

COMMENTS

Background

While once considered a benign process, nonalcoholic steatohepatitis (NASH) has been found to lead to cirrhosis and, in some cases, to hepatocellular carcinoma. The mechanisms underlying disease development and progression are awaiting clarification; however the finding that not all patients with steatosis develop hepatic inflammation and hepatocellular damage has led to the hypothesis that different pathogenic factors lead firstly to hepatic steatosis and secondly to hepatic damage ("the second hit"). Insulin resistance and obesity-related inflammation, among other possible genetic, together with oxidative stress, microcirculation disturbance, malnutrition are thought to play a key role in the second stage. People also found that small intestinal bacteria overgrowth (SIBO) may also play a role in the process of NASH. Some people think the medium of TNF-α to be an endotoxin, but all people do not accept this.

Research frontiers

Great effort has been and is still being done to clarify all the possible mechanism in the development of NASH, The source and pathogenesis of mediators of inflammation in the process has been the hotspot. People also want to know if it can act as a target point in the treatment of NASH.

Innovations and breakthroughs

In this article the relationship between the small bowel moment and SIBO is studied. And the cidomycin was first studied for the treatment of NASH.

Applications

This article helps to understand the process of SIBO leading to NASH, and suggests that Cidomycin might be useful in the treatment of NASH. Since there might be a vicious circle between SIBO and delayed intestinal motility, the treatment of accelerator of intestinal motility might also be useful.

Terminology

NASH: A liver disease, which resembles alcoholic liver disease consists of steatosis plus inflammation, necrosis, and fibrosis while lack of history of drinking. SIBO: Small intestinal bacterial overgrowth.

Peer review

This article tries to study the relationship between SIBO and delayed intestinal motility in NASH rats and suggest that cidomycin might be effective in the treatment of NASH. But the conclusion needs further probation in future clinical study before clinical application.

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CASE REPORT

Acute inflammatory demyelinating polyneuropathy associated with pegylated interferon α 2a therapy for chronic hepatitis C virus infection

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Abstract

The combination of pegylated interferon (Peg-IFN) and ribavirin is the standard of care for chronic hepatitis C virus (HCV) infection treatment. In general, common side effects related to this combination therapy are mild and are very well tolerated. However, peripheral neuropathy including demyelinating polyneuropathy related to Peg-IFN is extremely rare. We present the first case of an acute inflammatory demyelinating polyneuropathy (AIDP) associated with Peg-IFN-α 2a (Pegasys) after 16 wk of a combination therapy with Pegasys and ribavirin in a 65-year-old woman with chronic HCV infection. She developed tingling, numbness, and weakness of her upper and lower extremities and was hospitalized for acute neurological deficits. Her clinical course, neurological findings, an electromyogram (EMG), nerve conductions studies (NCS), muscle biopsy, and a sural nerve biopsy were all consistent with AIDP likely related to Pegasys use. The patient recovered completely with the use of intravenous immunoglobulin (IVIG) including physical therapy and neurological rehabilitation. It is very important that gastroenterologists and/or hepatologists recognize this rare neurological complication related to Peg-IFN treatment very early, since it requires a prompt discontinuation of therapy including an immediate referral to a neurologist for the confirmation of diagnosis, management, and the prevention of long-term neurological deficits.

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INTRODUCTION

The combination of Peg-IFN and ribavirin has been shown to be an effective treatment for chronic hepatitis C. Overall, the common side effects associated with these two drugs are well known. The common side effects of IFN include flu-like symptoms and psychiatric symptoms such as depression, suicidal ideation, irritability, nervousness, and insomnia[1-5]. Less common side effects include hematopoietic suppression, reversible hair loss, hearing loss, retinopathy, dermatitis, seizures, and the development or exacerbation of autoimmune diseases such as thyroid dysfunction, rheumatoid arthritis, sarcoidosis, systemic lupus erythematosus, vitiligo, type 1 diabetes, and myasthenia gravis[1-5]. Neurological complications, however, are rare. Only some publications reported on IFN-related peripheral neuropathy including sensory neuropathy[6,7]. In addition, rare neurological complications such as vasculitic neuropathy, autonomic neuropathy, Bell's palsy, and chronic inflammatory demyelinating neuropathy (CIDP) related to IFN use have also been documented in the literature[1,5,8,9]. Here we present the case of a severe AIDP that developed at wk 16 of Pegasys therapy.

CASE REPORT

A 65-year-old white female presented to liver clinic at the University of Chicago Medical Center for chronic hepatitis C treatment. The initial physical examination was unremarkable with the exception of spider angiomata in her neck and face, and bilateral palmer erythema. Initial liver profile included aspartate aminotransferase (AST) 56 (8-37) IU/mL, alanine aminotransferase (ALT) 67 (8-35) IU/mL,

total bilirubin 0.6 mg/dL, alkaline phosphatase 77 IU/mL, albumin 4.8 g/dL, and prothrombin time (PT) 13.1 s. Serum viral load was estimated to 2414896 IU/mL with a genotype 1a. A liver biopsy examination revealed a grade I and stage Ⅲ liver disease with a focal bridging fibrosis. The patient participated in a clinical trial for chronic hepatitis C and received Pegasys 180 μg subcutaneously per week and ribavirin 1000 mg/d in divided doses orally. HCV RNA levels decreased to 416 IU/mL after 12 wk of therapy, indicating an early viral response (EVR).

Approximately 13 wk after start of the treatment, the patient presented to the University of Chicago hospital with a 24 h onset of fever, chills, nausea, vomiting, headache, and fatigue. Her chest X-ray and CT scan of chest demonstrated a right upper lobe pneumonia. A cerebrospinal fluid (CSF) analysis revealed the presence of single WBC (monocyte), glucose levels of 74 mg/dL within the normal range, and a protein level of 15 mg/dL. However, her sputum culture grew pneumococci requiring treatment with vancomycin, ceftriaxone, and moxifloxacin for pneumonia. Pegasys and ribavirin treatment thus was temporarily discontinued. The patient was discharged home with oral moxifloxacin medication and recovered from pneumonia. However, shortly after discharge, the patient developed a macular rash on the anterior and posterior chest wall with an extension of rash into the anterior abdominal wall, which was attributed to the use of antibiotics. Two weeks after the onset of pneumonia (wk 15), the patient was restarted with Pegasys and ribavirin. Unfortunately, she reported tingling, numbness, and weakness of her lower extremities 3 wk later (wk 16). Her neurological symptoms progressed rapidly over the course of 1 wk to the point of losing the ability to ambulate, and she became wheelchair bound. In addition, the patient also complained of tingling and numbness in the upper extremities involving the fingers and wrists bilaterally. Pegasys and ribavirin were discontinued due to an acute onset of weakness of upper and lower extremities. The patient was hospitalized again. The neurological examination revealed normal cranial nerves with normal bulk of muscles and tone; but a weak muscle strength (4/5) in the upper extremities as well as in hips, knees, and ankle dorsiflexion and ankle plantarflexion bilaterally. Other muscle groups were even weaker (3/5) including ankles and toes. Tendon reflexes were diminished significantly. Sensory examination revealed decreased light touch, pinprick, and temperature sensation from the feet to mid-calves as well as hands except vibratory sense. A clinical diagnosis of acquired, acute demyelinating sensorimotor polyneuropathy was made. Other laboratory tests included a normal complete blood count, chemistry profile, erythrocyte sedimentation rate, and C-reactive protein. Antinuclear antibodies, rheumatoid factor, human immunodeficiency virus (HIV) test, anti-GM1 (ganglioside) antibodies, serum cryoglobulin level, Epstein-Barr virus serology, and cytomegalovirus serology were tested negative. A second CSF analysis was acellular again with protein (29 mg/dL) and glucose (60 mg/dL) levels within the normal range. In addition, myelin associated glycoprotein antibody was tested negative, and immunoglobulin A level was normal including negative immunoelectrophoresis for monoclonal antibodies in CSF.

Table 1 EMG/Nerve conduction studies

	Distal latency (m/s)	Conduction velocity (m/s)	Amplitude (uV (sensory) mV (motor))
Sensory			
Right sural	NR	NR	NR
Left sural	NR	NR	NR
Motor			
Right peroneal	8.9	27	0.6
Right tibial	9.5	27	1.4
Left tibial	7.9	30	1.1
Right ulnar	6	37	3.9
Right median	7.6	49	2.4
Left median	7.8	48	1.1
F wave	F-Latency		
Right tibial	Unclear		
Right ulnar	42.6		
Right median	54.8		
Left median	Unclear		

NR: No response obtainable.

An EMG, NCS, and Sural nerve biopsy were consistent with an AIDP (Table 1). The patient was treated with a course of IVIG at a dose of 0.4 g/kg per day. Unfortunately, she was only able to complete 4 d of treatment due to the development of a rash and leukopenia from IVIG. The patient was subsequently discharged for physical therapy and neurological rehabilitation where she had some improvement in her ability to write, to use utensils, and to ambulate with a walker.

Even though her neurological symptoms somewhat improved after a short course of IVIG, the patient developed bilateral foot drop, loss of finger grips, and a progressive weakness and numbness in both her hands and feet four weeks later. A neurological examination revealed decreased muscle bulks in the upper and lower extremities distally. Muscle strength was weaker (3/5) in finger abduction, plantar flexion and dorsiflexion bilaterally with bilateral foot drops. Tendon reflexes were 2/4 in the upper extremities but diminished significantly in the lower extremities, including absent reflexes at patella and achilles bilaterally. Remainders of motor examinations were normal. Sensory examination revealed normal sensation in the upper and lower extremities except absence of all modalities of sensation at her toes excluding sense of vibration and decreased pinprick sensation below the knees. Laboratory findings were again negative including anti- Sjogren's syndrome A (SSA) and Sjogren's syndrome B (SSB). Subsequently, a sural nerve biopsy and a gastrocnemius muscle biopsy were obtained and findings were consistent with an AIDP without showing any vasculitic features (Table 1). The patient was treated with 5 d of 2nd course of IVIG and the symptoms of weakness and bilateral legs numbness markedly improved over two months. Six months after the completion of the 2nd course of IVIG, the patient felt very well and neurological symptoms and signs resolved completely without having any residual neuromuscular deficits.

DISCUSSION

Peripheral neuropathy is a rare and uncommon side effect

seen in patients treated with IFN-α. In recent years, a variety of peripheral neuropathies have been reported in patients treated with IFN including sensory neuropathy, autonomic neuropathy, Bell's palsy, and more recently, CIDP[6-12]. As an example, one patient who received multiple cycles of IFN-α developed parasthesias of both legs and was diagnosed with sensorimotor polyneuropathy due to high cumulative effect of IFN-α[6]. IFN-α 2a has also been shown to have caused peripheral sensory neuropathy given for a period of twenty months[7]. In this case, when IFN-α 2a was discontinued for 4 wk, peripheral neuropathy resolved. However, upon reinitiating the IFN-α 2a, the peripheral neuropathy re-appeared suggesting IFN-related neuropathy. Furthermore, Lapinski *et al* reported a case of peripheral polyneuropathy in a patient who received Pegasys for 1 mo followed by pegylated IFN α-2b (Pegintron) for 3 mo that resulted in paresis of hands and legs leading to a CIDP confirmed by an EMG[9].

The more probable etiologies in our patient included infectious processes, toxins and/or drugs, and immune-mediated processes leading to demyelinating neuropathies[13]. Viral infections such as HIV and CMV have been described to cause demyelinating neuropathy[14,15]. HIV infection can lead to peripheral neuropathy that can rapidly progress to either acute or chronic demyelinating neuropathy due to macrophage-mediated immune attachment within the endoneurial parenchyma[16]. Since HIV test was negative in our patient this was not the cause of her neuropathy. A CMV infection in a patient with chronic HCV can present as an acute Guillain-Barre syndrome (GBS)[15]. It has been hypothesized that host immune system mistakenly attacks own nerve cells that expressed similar epitope as of HCV or CMV antigen. Our patient had multiple CSF analysis that failed to detect CMV DNA. Therefore, our patient also did not have CMV-related demyelinating neuropathy.

Toxins and/or drugs are also common etiologies for demyelinating neuropathies, which can be acute or chronic in nature. Common toxins/drugs are barbital, sulfonamides, phenytoin, nitrofurantoin, heavy metals, carbon monoxide, industrial poisons, and certain AIDS drugs (e.g., zalcitabine, didanosine)[13]. Our patient had no exposure to any of these above toxins or drugs other than Pegasys, ribavirin, and moxifloxacin. There are several reported cases of moxifloxacin induced peripheral sensory neuropathy including an evidence of a tendon rupture without showing demyelinating neuropathy[17]. Similarly, ribavirin also has not been associated with any type of reported neuropathy. But Pegasys including IFN-α has been implicated to cause immune mediated CIDP during chronic hepatitis C treatment[5,8] due to cytokine-induced apoptosis in the myelin-producing oligodendrocyte resulting in inhibition of central nervous system remyelination thus causing demyelinating neuropathy[5,18,19]. To the contrary, IFN-α has also been shown to be a successful treatment in patients with CIDP[8]. However, if a patient develops demyelinating neuropathy secondary to IFN use, it should be discontinued immediately since it may cause irreversible nerve damage due to inhibition of remyelination process. Two cases of CIDP have been described either with Pegasys or IFN-α use[5,8]. In both cases, parasthesia and muscle weakness developed 6 wk after therapy and lasted more than 8 wk suggesting CIDP. It has been shown that patients with CIDP respond to prednisone, plasma exchange, and IVIG[5,20,21]. The time course for our patient's symptoms was not long enough to meet the criteria for a CIDP. The criteria for CIDP usually include the clinical deterioration of neurological symptoms for a period of greater than 8 wk as opposed to AIDP and/or GBS, which usually has deterioration over a period of approximately 4 wk or less[21]. Furthermore, immune-mediated AIDP of GBS type has been reported after infection, post-vaccination, or surgery and also accounts for a significant portion of demyelinating polyneuropathies[13]. In our patient, there was an initial high suspicion for AIDP of GBS type due to a pneumonia prior to neurogical symptoms and signs. Her time course fit an acute demyelinating neuropathy given that it developed approximately 3 wk later. A typical presentation of GBS includes a significant motor weakness along with mild to moderate paresthesias. The muscle weakness typically starts in the distal muscles of the lower extremities and weakness ascends rapidly involving upper extremities. In addition, CSF analysis usually reveals an elevated protein level with normal white blood cell count. Even though our patient's presentation was suggestive of a GBS due to a preceding pneumonia, CSF analysis on multiple occasions revealed a normal CSF protein. Furthermore, our patient had significant painful paresthesias that are not commonly seen in GBS. Thus, the clinical course including normal CSF protein and painful paresthesias was not suggestive of a typical GBS, but rather suggestive of an acute immune mediated process related to Pegasys use as AIDP. The pathogenesis of AIDP related to Pegasys use is thought to be an acute immune-mediated progress similar to GBS. The precise and exact immune regulatory function of IFN-α including Peg-IFN is not well understood and it is likely similar to mechanism associated with IFN induced CIDP described above[5,18,19]. In addition, IFN-α has been reported to enhance *in vivo* and *in vitro* autoantibody production and may upregulate transcription of genes associated with class I major histocompatibility complex (MHC) antigens[22]. It is likely that the levels of proinflammatory cytokines may trigger autoimmune phenomena in immunologically predisposed individuals when IFN is administered. Therefore, the immune system mistakenly attacks the host's nerve tissue after recognizing a molecular epitope similar to a foreign antigen and may result in acute inflammatory neuropathy[23]. Immunomodulation with plasma exchange or IVIG usually shortens the disease process and provides the best outcome[23]. Our patient fit this clinical picture of an acute inflammatory neuropathy and showed a quick response to IVIG therapy.

One other possible immune-related process as the etiology of the demyelinating neuropathy would be IgM binding to Myelin-Associated Glycoprotein (MAG). Demyelinating polyneuropathy with monoclonal IgM is often associated with anti-MAG autoantibodies[24]. Our patient was negative for this antibody including negative immunoelectrophoresis suggesting this is not the case.

Vasculitic neuropathy, an autoimmune process may

also occur in association with chronic HCV infection. Generally, patients may develop a chronic symmetrical axonal sensorimotor peripheral polyneuropathy due to a necrotizing vasculitis[25]. Subsequent treatment with IFN-α can worsen vasculitic neuropathy[25]. In addition, there have been multiple reported cases of GBS associated with HCV related to cyroglobulinemia[26,27]. However, our patient had an undetectable cryoglobulin level on multiple occasions, which rules out a possibility of cryoglobulin induced demyelinating neuropathy.

In conclusion, the treatment of chronic HCV infection has come a long way in recent years. Physicians and other medical providers must be aware of possible side effects, including demyelinating neuropathy in patients who are treated with IFN and/or pegylated IFN in addition to HCV/cryoglobulin related neuropathy. As we learned from our case, Pegasys may cause neurological symptoms including AIDP. In addition, it is imperative on the part of the health care providers recognize AIDP very early for the diagnosis and treatment including the prevention of a long-term neurological complication by obtaining a neurology consultation.

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LETTERS TO THE EDITOR

Clinical guidelines: Involvement of peers increases physician adherence

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Abstract

The literature illustrates the important issue of physician adherence to guidelines in their daily practice. In a quantitative study, we asked a random sample of 100 hospital gastroenterologists to evaluate their knowledge of guidelines and awareness of promoters. The degree to which guidelines were considered reliable was not related to the scientific evidence but was significantly associated with the promoter. The French Society of Gastroenterology was considered to be a more reliable promoter than national health agencies and pharmaceutical industries. Gastroenterologists become aware of guidelines mainly through their specialty society (62%). Specialty societies appear to be a more important source of information on guidelines for physicians. National health agencies should involve the specialty societies in the guideline development process to achieve changes in clinical practice.

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TO THE EDITOR

Recently, Grassini *et al*[1] have shown that the percentage of inappropriate referrals for colonoscopy in an open-access endoscopy system is still high, despite the number of papers published on the issue and the definition of international guidelines. These results illustrate the important issue of physician adherence to guidelines in their daily practice.

In a study carried out in 2005, we showed how gastroenterologists judge and adhere to guidelines, based on who promotes them. Using a multiple-choice questionnaire, we asked a random sample of 100 hospital gastroenterologists to evaluate their knowledge of guidelines and awareness of promoters, and investigated how they became aware of the guidelines. The overall response rate was 71%. The degree to which guidelines were considered reliable was not related to the scientific evidence on which they were based (consensus conferences were considered more reliable compared to clinical practice guidelines 89.5% *vs* 77.6%, $P < 0.01$), but rather was significantly associated with the promoter. Specifically, the French Society of Gastroenterology was considered to be a more reliable promoter than national health agencies and pharmaceutical industries (67.4 *vs* 11.6 and 0.8%, $P < 0.001$).

Gastroenterologists become aware of guidelines mainly through their specialty society (62%), but also through congresses (31%), hospital colleagues (23%) and medical publications (14%). The main resources used for finding guidelines are the websites of specialty societies.

According to these results, peers and, in particular, specialty societies appear to be a more important source of information on guidelines for physicians. When previous studies have shown that the quality of some guidelines developed by specialty societies might be unsatisfactory, the authors have not criticized the specialty societies' influence for supporting guidelines[2,3]. Only the lack of explicit methodological criteria for production of guidelines was confirmed. Grol has recommended targeting each specific type of public for a best integration of the guidelines and an real impact in clinical practices[4]. Since many medical specialists (such as gastroenterologists, internists and primary care physicians) intervene in the field of gastroenterology, the target audience of the guidelines is very diverse and difficult to reach. National health agencies should integrate the specialty societies into the guideline development process to achieve change in clinical practice.

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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/eid.htm

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EDITORIAL

What's new in inflammatory bowel disease in 2008?

Daniel C Baumgart

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Abstract

Ulcerative colitis and Crohn's disease represent the two major forms of inflammatory bowel disease. In this highlight topic series of articles we cover the latest developments in genetics and epidemiology, intestinal physiology, mucosal immunology, mechanisms of epithelial cell injury and restitution, current medical therapy, modern surgical management, important extra-intestinal complications such as primary sclerosing cholangitis, cholangiocellular carcinoma and autoimmune hepatitis as well as endoscopic and molecular screening, detection and prevention of small bowel and colorectal cancer.

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Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Immunology; Genetics; Epidemiology; Medical therapy; Surgery; Cancer; Extra-intestinal manifestations

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In this special issue of the *World Journal of Gastroenterology* we have put together a group of expert faculty from all over the world to cover inflammatory bowel disease as a highlight topic.

Ulcerative colitis and Crohn's disease represent the two major forms of inflammatory bowel disease. Crohn's disease was first observed by the German surgeon Wilhelm Fabry (aka Guilhelmus Fabricius Hildanus) in 1623, and was later described by and named after the New York physician Dr Burril B Crohn[1,2]. Ulcerative colitis (UC) was first described by the London physician Sir Samuel Wilks in 1859[3].

Much has been learned since these early days about the etiology and particularly the genetic predisposition for these two disorders. The first paper in our series reviews the current knowledge about the etiology of inflammatory bowel disease. While all genome wide scans in the past have identified increased susceptibility genes, the most recently discovered susceptibility allele (IL23R) protects from Crohn's disease. The first article in our series puts this discovery in genetics in perspective with disease mechanisms and other etiological factors and its potential impact on the development of novel therapeutic strategies[4].

The most widely accepted hypothesis for the cause of inflammatory bowel disease is a disturbed interaction of the host immune system with the commensal microflora and other luminal antigens. This interaction begins at the epithelial layer and extends below to a multitude of other immune cells of the mucosal interface. Ultimately, mature and activated antigen presenting cells, (i.e. dendritic cells) induce and probably perpetuate an imbalance of effector and regulatory T-cell. Crohn's disease is a mainly a Th1 and Th17 mediated process, while ulcerative colitis appears to be predominately mediated through Th2 and NK T-cells. Two papers review the effects of these components in the immunopathogenesis of IBD[5,6].

Once the inflammation has occurred and is perpetuated by the dysfunctional immune system, the clinician is faced with distinct clinical phenotypes. Fortunately, the integrity of the gastrointestinal surface epithelium is rapidly reestablished even after extensive destruction. Rapid resealing of the epithelial barrier following injury is accomplished by a process termed epithelial restitution, followed by more delayed mechanisms of epithelial wound healing including increased epithelial cell proliferation and epithelial cell differentiation. Restitution of the intestinal surface epithelium is modulated by a range of highly divergent factors among them a broad spectrum of structurally distinct regulatory peptides, variously described as growth factors or cytokines[7].

Recent advances in our understanding of the pathophysiology of inflammation and in bioengineering have led to new therapeutic concepts targeting almost every aspect of the inflammatory process and help with the restitution of mucosal integrity. This provides clinicians with the opportunity of selecting from and combining conventional compounds and concepts as well as modern biologics and novel approaches to regimens tailored to the individual patient's needs[8].

Special issues in pediatric inflammatory bowel discussed are discussed in a separate paper[9].

Surgery has become an integral part of the management of inflammatory bowel disease and should not be considered a failure of medical management. Modern surgical techniques including minimally invasive procedures and current issues of surgical management are reviewed[10].

Inflammatory bowel disease is a systemic illness, not limited to the gastrointestinal tract. A substantial fraction of patients develops extra-intestinal manifestations, which itself can cause additional morbidity and complications. Hepatic manifestations and especially primary sclerosing cholangitis are among the most common, sometimes overlap and cause diagnostic and therapeutic challenges[11].

Finally, patients with chronic inflammatory conditions, including inflammatory bowel disease are prone to develop malignant complications. The final paper reviews current knowledge about the incidence and prevention of cancer[12].

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TOPIC HIGHLIGHT

Daniel C Baumgart, MD, PhD, *Series Editor*

Primary sclerosing cholangitis, autoimmune hepatitis and overlap syndromes in inflammatory bowel disease

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Abstract

Primary sclerosing cholangitis (PSC) is a chronic progressive disorder of unknown aetiology characterised by chronic inflammation and stricture formation of the biliary tree. Symptoms include itch and lethargy and in advanced cases cholangitis and end-stage liver disease, however increasing numbers of asymptomatic individuals are being identified. The disease is rare in the general population but is strongly associated with inflammatory bowel disease (IBD) affecting up to 5% of patients with Ulcerative Colitis, with a slightly lower prevalence (up to 3.6%) in Crohn's disease. The strength of this association means that the vast majority (> 90%) of patients with PSC also have IBD, although many may have only mild gastro-intestinal symptoms. Usually IBD presents before PSC, although vice-versa can occur and the onset of both conditions can be separated in some cases by many years. Mean age of diagnosis of PSC is in the fifth decade of life with a strong male predominance. Risk is increased in those with a family history of PSC, suggesting a genetic predisposition and the disease is almost exclusive to non-smokers. The ulcerative colitis associated with PSC is characteristically mild, runs a quiescent course, is associated with rectal sparing, more severe right sided disease, backwash ileitis and has a high risk of pouchitis post-colectomy. Most worrisome is the high risk of colorectal malignancy which necessitates routine colonoscopic surveillance. Cholangiocarcinoma is also a frequent complication of PSC with a 10%-15% lifetime risk of developing this condition. Treatment with high dose ursodeoxycholic acid offers some chemoprotective effects against colorectal malignancy and may decrease symptoms, biochemical and histological progression of liver disease. Small duct PSC patients characteristically have normal cholangiography, and liver biopsy is required for diagnosis, it appears to have a more favourable prognosis. Autoimmune Hepatitis (AIH) is also more prevalent in patients with IBD, with up to 16% of patients with AIH also having ulcerative colitis. A small subgroup of patients have a AIH-PSC overlap syndrome and the management of these patients depends on liver histology, serum IgM levels, autoantibodies, degree of biochemical cholestasis and cholangiography as some of these patients may respond to immunosupression.

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Key words: Primary sclerosing cholangitis; Autoimmune hepatitis; Liver disease; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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INTRODUCTION

The first association between colonic ulceration and liver disease was made in 1874 by Thomas CH, who described a young man who died of a 'much enlarged, fatty liver in the presence of ulceration of the colon'[1]. The association was confirmed by James Lister in 1899, who reported a patient with ulcerative colitis and secondary diffuse hepatitis[2]. Over the next 100 years it has become well established that there is a close relationship between inflammatory bowel disease and various hepatobiliary disorders. These disorders are listed in Table 1.

PRIMARY SCLEROSING CHOLANGITIS

First described by Smith and Loe in 1965[3] Primary sclerosing cholangitis (PSC) is a chronic progressive disorder of unknown aetiology characterised by inflammation, fibrosis and stricture formation in medium and large sized ducts in the biliary tree[4,5]. Common symptoms include itch and lethargy although many patients (up to 45%) are asymptomatic, even with advanced disease[5]. The disease is strongly associated with Inflammatory Bowel Disease and may be detected as an incidental finding of a raised serum alkaline phosphatase. Magnetic resonance

Table 1 Hepatobiliary disorders associated with inflammatory bowel disease

Hepatobiliary disorders	Associated with	
	Ulcerative colitis	Crohn's disease
Primary sclerosing cholangitis (PSC)		
Large duct PSC	+	+
Small duct PSC ('pericholangitis')	+	+
Cirrhosis	+	+
Hepatoma	+	+
Cholangiocarcinoma	+	+
Miscellaneous disorders		
Fatty liver	+	+
Granulomas		+
Amyloidosis		+
Hepatic abscess		+
Gallstones		+
Autoimmune hepatitis	+	
Primary biliary cirrhosis	(+)	
Budd-Chiari syndrome	(+)	(+)

+: Definite association; (+): Possible association.

Table 2 Prevalence of primary sclerosing cholangitis in patients with ulcerative colitis

Country of origin	Number of patients with ulcerative colitis	Percentage with primary sclerosing cholangitis
Oxford, UK[9]	681	2.9
Oslo, Norway[12]	336	4
Stockholm, Sweden[11]	1500	3.7 (5.5 in total colitis)

cholangography (MRCP) and endoscopic cholangiography (ERCP) are diagnostic, demonstrating the diffuse multifocal strictures and dilatation giving rise to a characteristic 'beaded' appearance (Figure 1).

Prevalence

PSC is rare in the general population. Data from Olmsted County (USA) in the year 2000 identified 20.9 cases of PSC per 100 000 of the population in men and 6.3 per 100 000 in women[6], which is similar to that seen in the UK[7]. There has been a suggestion that the incidence may be rising. In Spain the prevalence in 1988 was 2.24 per million having been 0.78 cases per million in 1984[8]. It is however unclear if this represents a true increase in the disease or improved case recognition due to improved diagnostics particularly in asymptomatic individuals.

PSC in IBD

Because of its strong association with inflammatory bowel disease (IBD) PSC is far more common in patients with ulcerative colitis (UC). The prevalence quoted in different case series varies widely, largely due to differences in the number of patients included with severe, active, or extensive inflammatory bowel disease studied and also in the method used to investigate liver dysfunction. Studies on unselected patients provide the most accurate data but are ethically difficult as both liver biopsy and ERCP are associated with significant morbidity. In an early study of unselected adult patients from Oxford, 5%-6% of the 300

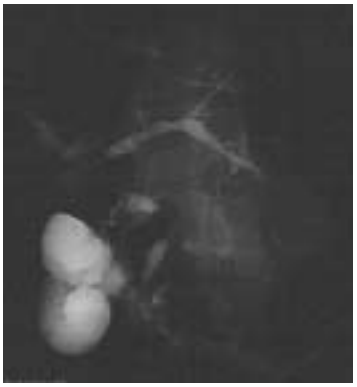


Figure 1 Endoscopic Retrograde Cholangiogram showing the classic 'beaded' appearance of bile ducts in Primary Sclerosing Cholangitis.

patients with ulcerative colitis had significant histological abnormalities on hepatic histology compared with 10% of 100 unselected patients with Crohn's disease; none of these patients underwent cholangiography[9,10]. Studies (Table 2) using selected patients include a large Swedish study of 1500 patients with UC where it was found that 5% of patients had increased serum Alkaline Phosphatase and, of those who underwent ERCP, 85% had evidence of PSC, being more common in men and those with pancolitis[11]. Other reports have found similar results. In a study from Oslo, 14% of patients had evidence of hepatobiliary pathology with 4% of patients with UC having PSC[12]. Thus it is generally accepted that approximately 5% of patients with UC will have associated PSC.

The percentage of Crohn's disease patients affected by PSC is much smaller[13]. In one study of 262 patients, 38 (15%) had abnormal liver function tests and underwent ERCP and liver biopsy, 9 of these (3.4% of the total) had PSC[14]. Crohn's disease associated with PSC is generally seen in patients who have extensive colonic or ileocolonic disease, or in paediatric populations.

IBD in PSC

Vice versa the great majority of patients with PSC have underlying IBD. The frequency with which it is recognised again varies widely depending on the intensity with which it is sought, varying from a minimum 25% of all PSC patients having IBD if identified by clinical history alone to 90% of patients if biopsies of rectum and sigmoid are taken at the time of flexible Sigmoidoscopy[15]. There are also large geographical variations in the prevalence of IBD in PSC (Table 3). Since the recognition of inflammatory bowel disease has important implications in terms of risk of colorectal cancer (PSC patients without IBD are not at increased risk of colorectal cancer and do not require colonoscopic surveillance[16]) it is the authors practice to perform colonoscopy and serial biopsies in all patients with newly diagnosed PSC.

Aetiology

The aetiology of PSC itself is unknown. The 2:1 male to female gender ratio of patients and the relatively poor response of the disease to immunosuppression suggest that PSC is not a classic autoimmune disease. However the association of PSC with IBD, autoimmune diseases, and a host of other humoral and cellular immune abnormalities

Table 3 Prevalence of inflammatory bowel disease in patients with primary sclerosing cholangitis (PSC)

Institution (country of origin)	Number of patients with PSC	Percentage with IBD
Royal Free Hospital (UK)[17]	29	72
Mayo Clinic (USA)[18]	50	70
Huddinge (Sweden)	305	72
King's College London (UK-children)	13	77
Okolicsanyi (Italy)[19]	82	54
Escorsell (Spain)	43	47
Okada (Japan)[20]	155	23

(Table 4) make an immunopathogenic mechanism likely. Particularly common serological abnormalities are raised IgM levels in up to 50% and positive anti-smooth muscle antibodies and antinuclear antibodies in up to 75%[21]. Anti Neutrophil Cytoplasmic Antibodies with a pericellular pattern (pANCA) are also a common finding, being present in 80% of patients being and unrelated to the presence or absence of UC. Cellular immunity anomalies include portal tract infiltration of functional T cells, restricted T cell receptor repertoire, and aberrant expression of HLA and costimulatory molecules on biliary epithelial cells.

Immunogenetic studies have identified a number of key HLA haplotypes (Table 5) associated with PSC, HLA-B8/DR3 haplotype being particularly common in patients with PSC and UC and infrequent in patients with PSC alone[22]. This haplotype is also associated with other organ-specific autoimmune disease including coeliac disease, thyrotoxicosis, lupoid autoimmune hepatitis, myasthenia gravis, and type 1 diabetes mellitus. However PSC, independent of HLA type, is also associated with a range of autoimmune diseases, diabetes mellitus and Graves disease being the most common. Saarinen *et al* found that 25% of patients with PSC had one or more autoimmune disease, compared to 9% of patients with inflammatory bowel disease alone[23].

Possible explanations for the PSC association with IBD include the development of autoantibodies to an unknown antigen in an immunogenetically susceptible host which cross reacts with biliary and colonic epithelium and is capable of inducing complement activation[24]. Alternatively, the initiation of the immune response may be the ingress of bacteria or other toxic metabolites through the diseased bowel wall.

CLINICAL COURSE

Onset

PSC is a disease of middle age with a mean age of diagnosis is 40 years in men and 45 years in women. Bowel symptoms have usually developed before those of PSC and UC is therefore usually diagnosed several years before PSC. However occassionally symptoms of PSC may precede IBD by up to 4 years[25]. The age of onset of the symptoms also has important implications as there is evidence to suggest patients with Colitis in childhood have a high prevalence

Table 4 Evidence for the influence of immune mechanisms on the aetiology of PSC

Humoral immunity	Increased circulating immune complexes
	Elevated immunoglobulin levels (IgG and IgM)
	Low titres of non-organ specific autoantibodies (ANA and SMA)
	High titres of antineutrophil antibodies
	Autoantibodies to BEC surface antigens
Cell mediated immunity	Decreased levels of circulating peripheral CD8+ve Tcells
	Portal T cell and NK cell infiltrate
	Increased activated and memory T cells
	Restricted T cell receptor repertoire (Vβ3)
	Aberrant expression of HLA-DR on BEC
	Coexpression of costimulatory molecules and HLA-DR on BECs
	Increased circulating and tissue bound adhesion molecules
Immune effector mechanisms	Enhanced cytokine expression in the liver
Immunogenetic mechanisms	HLA associations

Table 5 Key HLA haplotypes associated with primary sclerosing cholangitis

Haplotype	Significance in PSC
B8-TNF*2-DRB3*0101-DRB1*0301-DQA1*0501-DQB1*0201	Strong association with disease susceptibility
DRB3*0101-DRB1*1301-DQA1*0103-DQB1*0603	Strong association with disease susceptibility
DRB5*0101-DRB1*1501-DQA1*0102-DQB1*0602	Weak association with disease susceptibility
DRB4*0103-DRB1*0401-DQA1*03-DQB1*0302	Strong association with protection against disease
MICA*008	Strong association with disease susceptibility

of liver abnormalities than adults, with up to 60% children having abnormal liver function tests[26].

Risk factors

Unlike other immune mediated diseases PSC is far more common in men with 70% of all PSC patients being male. This male preponderance is absent in PSC patients without associated IBD, being equally common in both males and females[27]. In addition to IBD and male sex, another recognised risk factor is a family history of the disease, There is a 0.7% risk in first degree relatives of patients with PSC rising to 1.5% in siblings of affected individuals[28].

Smoking is a well recognized protective factor against the development of ulcerative colitis, and three studies have suggested that cigarette smoking may also additionally protect against the development of PSC. Moreover, this protective effect was more marked in patients with primary sclerosing cholangitis than ulcerative colitis and was seen in patients with and without inflammatory bowel disease[29-31]. The mechanism of protection in both disorders remains unknown.

Clinical characteristics of IBD associated with PSC

There are a number of unique features of Ulcerative

Colitis associated with PSC (Table 6). The colitis is often mild, asymptomatic and runs a quiescent course[32]. It is associated with rectal sparing[33]; and therefore not seen on rigid sigmoidoscopy; is often more severe proximally and associated with backwash ileitis. It may be visible microscopically with a relatively normal macroscopic appearance and pouchitis is far more common after colectomy and ileo-anal pouch formation, occurring in up to 60% of PSC patients compared to 15% in patients with UC alone[34].

Most importantly PSC is a significant risk factor for the development of carcinoma of the colon in patients with inflammatory bowel disease[35-37]. A meta-analysis demonstrated a four fold increase in colon cancer in UC patients with PSC when compared to UC alone (RR 4.26 95% CI 2.8-6.48)[38]. The risk of proximal cancer is particularly increased[37,39]. Additional risk factors are duration and extent of disease and a family history of colorectal cancer[40]. Current U.K. guidelines therefore recommend annual screening colonoscopy in IBD patients with PSC[41].

Treatment of PSC with ursodeoxycholic acid appears to have a chemopreventative effect decreasing the incidence of carcinoma of the colon and is therefore recommended[42]. Treatment with 5-aminosalicylic acid (5-ASA) compounds have also been shown to reduce the risk of colorectal cancer in general patients with UC[43]. There are no studies specific for PSC/IBD patients in this context but it is reasonable to infer that it will be protective and is also therefore recommended.

Prognosis

Despite treatment however PSC is generally progressive and prognosis poor. Median survival without liver transplantation after diagnosis is approximately 12 years[44] with survival worse for those who are symptomatic at presentation[45].

The major cause of mortality is the occurrence of cholangiocarcinoma, which is significantly increased in patients with PSC who display a 10%-15% lifetime risk of developing the disease. This risk is higher in patients with associated inflammatory bowel disease than those with PSC alone, with an estimated annual incidence of 0.5%-1%[46-48]. Unfortunately there remains no validated screening tool for cholangiocarcinoma and therefore tumours often present at an advanced stage and have a poor prognosis. Other complications of PSC include osteoporosis, this is a frequent finding the mechanism of which remains unclear[49]. Bone density measurement should therefore be considered in these patients.

PSC treatment

Treatments which have been tried in an attempt to prevent progression of the liver disease to cirrhosis and to decrease the risk of complications especially malignancy include tacrolimus[50], methotrexate[51], corticoteroids[52], D-penillamine[53], etanercept[54], cyclosporin[55], azathioprine, 6-mercaptopurine[56] and pentoxifylline[57]. None of these agents have demonstrated any consistent benefit.

Ursodeoxycholic acid (UDCA) is the only drug which has been demonstrated to have a positive effect. It is also the most extensively studied[58] with some, but not all, studies showing improvements in biochemistry, symptoms and histology[59-64]. The most promising data is for long term UDCA used at high dose, 20-30 mg/kg per day (in divided doses), as this may increase clinical benefit and decrease histological progression of disease[65,66]. In view of its chemopreventative effect against colon cancer and its low incidence of side effects, high-dose UDCA is currently the best available treatment for PSC.

Table 6 Clinical, Endoscopic, and Histological findings that characterize IBD-PSC

Male predominance
Quiescent colitis
Pancolitis
Rectal sparing
Backwash ileitis
Pouchitis
Colorectal dysplasia/carcinoma
Different extracolonic manifestations

The concurrence of both conditions also requires that the impact of treatment of either disease on the other must be considered. Proctocolectomy appears to have no effect on liver biochemistry, histology or survival of patients with PSC and chronic UC[67]. However the natural history of UC following liver transplantation is variable[68-72]. One study showed approximately a third had improvement in colitis, a third remained unchanged and a third suffered increased activity of colonic disease[73]. Several series report increased rates of colon cancer and colectomy after liver transplantation[74-76]. It remains unclear if this is due to longstanding immunosuppressant use or confounding variables such as long duration of disease.

SMALL DUCT PSC

A variant of the disease called 'small duct PSC' is applied to the small percentage of patients with characteristic clinical findings but who have a normal cholangiogram. The absence of macroscopic abnormalities necessitates liver biopsy for the diagnosis of small-duct PSC. Characteristic features of PSC on liver biopsy are concentric rings of connective tissue around bile ducts, the so-called 'onion ring' appearance[77]. Other features which are less specific include enlargement of the portal triads, periportal inflammation with mononuclear and polymorphonuclear cells, oedema and bile duct proliferation. Extension into the hepatic parenchyma with bridging fibrosis and ultimately cirrhosis represents advanced disease.

Small duct PSC appears to have a more favourable prognosis with a median survival 29.5 years compared to 17 years for classical PSC[78-81], although occasionally patients with small duct PSC progress to large duct disease.

AUTOIMMUNE HEPATITIS AND OVERLAP SYNDROMES IN IBD

Patients with IBD are also at increased risk of other of

immune mediated liver disease including autoimmune hepatitis (AIH). In one study, UC was present in 16% of patients with AIH[82]. AIH and PSC may also occur within the same individual and it remains unclear if this represents the independent occurrence of both diseases, either sequentially or concurrently, the presence of a distinct overlap syndrome or different stages in the evolution of a single disease entity.

Difficulties with definitions and thus classification of these patients has led to widely varying estimates as to the frequency of the coexistence of the two conditions with studies ranging from 7.6% to 53.8%. of patients with PSC have features of AIH[83-89]. and conversely an abnormal cholangiogram being present in 42% of patients with AIH and UC. The association between AIH and PSC appears more common in the paediatric population.

Patients with PSC should be suspected of having associated AIH or AIH-PSC overlap if they have interface hepatitis on biopsy, high serum IgG levels, lower ALP and the presence of autoantibodies (ANA or SMA titre > 1:40). It is important to identify these patients as they maybe responsive to treatment with steroids[85].

Patients with AIH should be suspected of having associated PSC or AIH-PSC overlap[90] if they have pruritis, UC, bile duct abnormalities on histology, cholestatic liver biochemistry (ALP × 2 ULN) and abnormal cholangiography. These patients tend to be unresponsive to steroids[87,91-93].

CONCLUSION

From the original descriptions of liver disease associated with disease of the bowel over a century ago much progress has been made. The association of inflammatory bowel disease with PSC, AIH and overlap syndromes is now well established and much is known on the epidemiology and course of the conditions. Although there remain many more questions than answers, an increasingly developed concept of hepatobiliary disorder in inflammatory bowel disease has emerged. It is suggested that the major hepatobiliary diseases seen in association with both UC and Crohn's disease, namely PSC, cirrhosis, cholangiocarcinoma, and most cases of autoimmune hepatitis, represent different aspects of the same spectrum of hepatobiliary disease. However what is cause, what is consequence and what is coincidence remains to be seen.

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TOPIC HIGHLIGHT

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Inflammatory bowel disease: Genetic and epidemiologic considerations

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Abstract

Genome-wide association studies have firmly established that many genomic loci contribute to inflammatory bowel disease, especially in Crohn's disease. These studies have newly-established the importance of the interleukin 23 and autophagy pathways in disease pathogenesis. Future challenges include: (1) the establishment of precisely causal alleles, (2) definition of altered functional outcomes of associated and causal alleles and (3) integration of genetic findings with environmental factors.

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Key words: Crohn's disease; Ulcerative colitis; Interleukin 23; Autophagy; Complex genetics

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INTRODUCTION

The fields of genetics and epidemiology share a common goal of identifying specific factors, present in some individuals, and absent in others, that contribute to disease causation. As with many diseases, in inflammatory bowel disease (IBD), carefully performed epidemiologic studies provide an ongoing basis for the rationale and design of genetic studies. Observations on the nature and magnitude of familial clustering of IBD cases provided the framework for the present genetic observations that Crohn's disease (CD) and ulcerative colitis (UC) share some susceptibility alleles (e.g. *IL23R*) whereas others are unique to one disease subtype or the other. This review summarizes present understanding of the genetics and epidemiology of IBD, and describes the encouraging early results from genome-wide association studies which demonstrate significant consistency between studies.

EPIDEMIOLOGIC OBSERVATIONS FORM THE BASIS FOR GENETIC STUDIES IN IBD

Familial risk in IBD

The observation that cases of IBD cluster within families suggested that both shared genetic and environmental factors could contribute to disease pathophysiology. That monozygotic (MZ) twin concordance is significantly higher than dizygotic (DZ) twin concordance for both CD (20%-50% *vs* 0%-7%, respectively) and UC (14%-19% *vs* 0%-5%, respectively) indicates that genetic factors definitively contribute to both disorders[1-4]. The observation that, even among MZ twins, disease concordance is significantly less than 100% demonstrates the significant role that environmental and developmental factors play in disease pathogenesis. Like most genetic disorders, inheritance in IBD does not follow a simple Mendelian pattern. This would indicate that no single gene mutation is sufficient and/or necessary to contribute to disease, indicating that IBD represents complex, multigenic genetic disorders. In complex genetic disorders, any single gene mutation/polymorphism will not confer a simple determinative effect of disease development (as is the case for Mendelian, single-gene disorders), but rather merely confers a statistically increased, but non-determinative susceptibility to developing disease. Approximately 5% to 20% of patients with IBD have a family history of IBD[5,6]. A family history of IBD is generally more prevalent among CD than UC patients[6]. The relative risk to siblings, λs, (sibling risk compared to general population risk) ranges from 30-40 for CD and 10-20 for UC[7,8]. Furthermore, the relative risk for UC to siblings of a CD proband was observed to be 3.9 and the cross-disease relative risk to siblings of a UC proband was observed to be 1.8, suggesting that CD and UC are genetically related[9].

Comparative prevalence of IBD

The prevalence of CD and UC is highest in North America, northern Europe, and the United Kingdom, with averages ranging from 100 to 200 cases per 100 000[10-13]. IBD is more common in European Americans compared with African Americans, and the lowest rates of IBD have been reported in Hispanics and Asians[7,14-17]. The

prevalence of CD based on inpatient and outpatient visits in a Southern California Kaiser HMO study was 43.6 per 100 000 for European Americans, 29.8 per 100 000 for African-Americans, 5.6 per 100 000 for Asians and 4.1 per 100 000 for Hispanics[18]. However, two different Kaiser HMO studies found similar rates of hospitalizations among African-American and European ancestry individuals[18,19]. In addition, a more recent study demonstrated a similar incidence of IBD in African-Americans and European Americans[20]. Within European ancestry populations, the rates of IBD are higher in persons of Jewish ancestry than other ethnic groups[14,21], and higher in Ashkenazi (Central and Eastern European) Jews than in Sephardic (Middle Eastern and Spanish ancestry) Jews[14,21].

The hygiene hypothesis

Generally, IBD incidence is lower in developing countries and higher in industrialized countries, such as in North America and Europe; as a country undergoes the transition to industrialization, the prevalence of UC rises first, followed by a rise in the prevalence of CD[10]. The hygiene hypothesis proposes that lack of exposure to select microbial agents in childhood causes IBD[22]. In support of this, in Manitoba, patients with CD are less likely to have lived on a farm and less likely to have drunk unpasteurized milk. Furthermore, CD patients were more likely to have used tap water rather than well water, and more likely to have had a pet[23]. Against this hypothesis, a separate study of pediatric IBD observed that owning a pet, day-care attendance, and "physician diagnosed infection" between 5 and 10 years of age was associated with increased CD risk, and regular use of a personal towel and lesser crowding in the home with decreased risk[24].

The measurable changes in disease incidence in various geographic regions highlights the contributory role of environmental factors in IBD pathogenesis. A leading culprit for such environmental contributions is a changing intestinal intraluminal microbial milieu. At birth, the intestinal lumen is largely sterile[25], with rapid bacterial colonization ensuing in the neonatal period. There is some evidence to suggest that fecal colonization patterns may reflect environmental cues in the perinatal period and thus may be manipulable[26,27] as a potential means of preventing disease in high risk individuals.

Tobacco and IBD

Within Western countries, smoking is the most well established environmental risk factor, increasing risk for CD by approximately two-fold[28] and non-smoking decreasing risk for UC two- to five-fold[14]. Being an ex-smoker increases the risk of developing UC two-fold[29]. Ex-smokers make up an increasing percentage of older patients diagnosed with UC, accounting for more than 35% of the attributable risk of late onset (> 45 years) UC and a large component of the second peak in diagnosis[30]. Interestingly, Bridger *et al* observed that in IBD pedigrees with UC/CD sibling pairs, smokers tend to develop CD and non-smokers UC (O.R. 10.5)[31]. A significant increase in smoking in younger patients with familial CD has been reported[30]. Smoking is also associated with early recurrence of CD following surgery[32]. Future genetic studies in IBD will explore whether specific genetic associations will be stratifiable based on tobacco status at the time of diagnosis. However, tobacco may exert many of its phenotypic effects through epigenetic (reversible, changes in gene regulation that occur without a change in DNA sequence or genotype) mechanisms, such as DNA methylation effects on transcriptional activation[33].

HUMAN GENETIC VARIATION AND THE HAPMAP PROJECT

The most abundant of the human genetic variants are single nucleotide polymorphisms (SNPs). A SNP is a DNA sequence variation occurring when a single nucleotide, A, T, C, or G, in the genome differs between individuals, or between homologous chromosomes within an individual. Three salient features of SNPs include, (1) their allele frequencies within a population of interest, (2) their correlation, or linkage disequilibrium, with SNPs in the immediate genomic vicinity, and (3) whether or not the SNP of interest results in a measurable phenotypic change in protein function and/or expression, that is, represents a functional polymorphism.

As a very general rule, SNPs with a minor allele frequency approaching 50%, are genetically more ancient, having had a longer time to increase in frequency, and are more likely to be observed in all racial groups. In contrast, uncommon SNPs having a minor allele frequency of less than 5% (that is 5 of 100 chromosomes tested from 50 individuals carry the minor allele) are more likely to be evolutionarily more recent, and are often observed uniquely in one racial group or another. A number of factors besides the evolutionary age of a SNP can affect its frequency within a population, notably whether or not the SNP confers a selective advantage or disadvantage with respect to reproductive fitness within a population. It may be speculated that functional SNPs that contribute to the development of chronic inflammatory disorders such as IBD may confer a selective advantage with respect to combating historically significant infectious pathogens.

It is estimated that there are 5 million SNPs common SNPs having a minor allele frequency greater than 10%[34]. In order to comprehensively sample all common variation throughout the genome, however, it is not required that millions of SNPs be directly tested, due to the high degree of correlation, or linkage disequilibrium that exists between SNPs throughout the human genome. The Human HapMap Project empirically defined the linkage disequilibrium patterns in European, African and Asian cohorts and provided the basis for the genome-wide association studies that increasingly being reported for various complex genetic disorders[35]. It is estimated that genotyping several hundred thousand SNPs in European ancestry cohorts will sample nearly 80% of the common human variation with an r^2 (measure of linkage disequilibrium) of greater than 0.8[36].

The costs of genotyping several hundred thousand SNPs continue to decrease, and therefore genotyping sufficiently large cohorts to identify IBD genes through genome-wide association studies is now feasible. Once

associations with SNPs are identified, the challenge remains to identify the functional polymorphisms that account for the statistical association. The precedent from Mendelian disorders would suggest that those amino acid variants which directly affect protein structure are likely to disproportionately contribute to disease susceptibility. Within amino acid polymorphisms, those variants in highly conserved (between species) regions or contained within key functional domains are more likely to have significant functional effects. Ultimately, however, proof of disease contribution requires demonstration of altered functional effects associated with the mutation of interest.

IBD GENETIC ASSOCIATIONS PRIOR TO THE ADVENT OF GENOME-WIDE ASSOCIATION STUDIES

Because the statistical effects observed in multigenic disorders are relatively modest, the hallmark for accepting genetic associations is replication in independent cohorts. However, interpretation of disease association studies has been complicated by the reporting of often conflicting studies. In such instances, meta-analyses of all reported studies can provide some insight, with the caveat that publication bias (e.g. negative studies may be more difficult to publish than positive ones) may occur.

Nod2 (CARD15) associations to ileal CD

The most well-replicated IBD genetic associations is the Nod2 gene association with ileal CD[37,38]. Nod2 was the first definitive risk factor for CD and one of the first genes identified for a common complex genetic disorder. It functions as an intracellular sensor for bacterial peptidoglycan[39], and can be activated by a minimal bioactive component, muramyl dipeptide (MDP)[40,41]. MDP is present in both gram positive and negative bacteria and activates NF-κB and MAP kinase pathways[42]. Three uncommon (minor allele frequency less than 5% in healthy controls) polymorphisms, Arg702Trp, Gly908Arg, and Leu1007fsinsC are each highly associated with CD. These findings have been extensively replicated by a number of subsequent studies. A meta-analysis of 39 studies showed an odds ratio for simple heterozygotes of 2.4 (confidence interval, C.I. 2.0-2.9), and for homozygous/compound heterozygous carriers of 17.1 (C.I. 10.7-27.2)[43]. Nod2 carriage has been specifically associated with ileal involvement, stricturing complications and a modestly earlier age of onset.

Each of the three CD polymorphisms are located within or near the leucine rich repeat, sensing domain of Nod2, and each results in a decreased capacity to activate NF-κB in response peptidoglycan or MDP stimulation[39,44-48]. The frameshift mutation, Leu1007fsinsC demonstrates a largely complete deficiency in the capacity to signal in response to MDP stimulation[45]. Therefore, these functional polymorphisms represent direct risk alleles for CD. Importantly, the Nod2 discovery provides specific support for the long-held hypothesis that CD results from a genetically dysregulated host immune response to luminal bacteria. These Nod2 mutations have not been observed in Japanese, Chinese and Koreans with IBD[49-51], and they are rare in African-American IBD[52].

Despite the high odds ratios associated with the Nod2 mutations, it is estimated that the disease penetrance, even for homozygous or compound heterozygous carriers is limited, suggesting that these Nod2 variants alone are insufficient to produce disease. As each of the three major mutations demonstrates decreased function in primary human cells[39,44-48], Nod2-deficient murine models provide an important model for human disease. The absence of intestinal inflammation in Nod2-deficient mice further highlights the insufficiency of this pathway alone to induce CD[42,53]. Since the discovery of the CD-association with Nod2, a number of studies have added to understanding of the Nod2 functional pathway.

Given the dysregulation in intestinal immune homeostasis characteristic of CD, it is logical to hypothesize that Nod2 is important for either impaired tolerance mechanisms critical in limiting excessive activation of the intestinal immune system and/or altered initial defenses against intestinal bacteria. There are a broad range of tolerance mechanisms operating in the intestine to regulate excessive immune responses in the context of its unique exposure to a continuous bacterial load. Regarding initial defenses, a defect in bacterial recognition and responses at the intestinal epithelial surface might result in alterations in the population of commensal organisms, and/or increased invasion, which in turn, could contribute to an increased propensity toward intestinal inflammation. Nod2 is highly expressed in Paneth cells of the small intestine[54,55] and Nod2 mutations are associated with ileal location of disease[56]. Studies in humans and mice have shown that normal Nod2 function is required for optimal defensin expression and therefore, impaired defensin expression and regulation may contribute to the increased CD susceptibility observed in Nod2 risk allele carriers[57-59].

IBD5 association on chromosome 5q31

Within the IBD5 linkage region, association of CD with a 250 kb region on chromosome 5q31 was reported[60]. This association has been subsequently replicated in a number of studies[61-64], for both CD and UC[62,65]. Candidate polymorphisms found on this haplotype within the organic cation transporter OCTN1 (*SLC22A4*) and OCTN2 (*SLC22A5*) genes have been reported[66,67]. However, statistically equivalent evidence for association has been observed throughout the risk haplotype[68] encompassing several genes, highlighting the need for additional genetic and functional investigation in this region. Phenotypic correlates have been reported for perianal disease[61,69], colonic location[70], disease complications and progression[68,71], extensive disease in UC[72], as well as for decreased height and weight at diagnosis in pediatric cohorts[73]. In contrast, some studies have not observed clear genotype-phenotype correlations[74,75].

HLA associations

Replicated HLA class Ⅱ associations in IBD include HLA-DRB1*1502 (serological marker HLA-DR2)[76] association with UC, and HLA-DRB1*0103 association with UC and colonic CD[77,78]. HLA-DRB1*0103 is noteworthy in that

it is a risk factor for both UC and colonic CD, suggesting it may play a role in chronic inflammation of the colon independent of major IBD phenotype (i.e., CD or UC)[78]. As with IBD5, the DRB1*0103 and DRB1*1502 class Ⅱ variants are in strong linkage disequilibrium with SNPs on multiple immunologically active candidate genes. Present approaches have not been able to discern whether these other genes or the class Ⅱ genes are the true risk genes in the HLA locus.

Reported, but unconfirmed IBD associations

Associations which have been reported within linkage regions to IBD but have not been consistently replicated include an indel polymorphism in the promoter region of the *NF-κB1* gene on chromosome 4q[79-82], multiple polymorphisms in the *MDR1/ABCB1* gene on chromosome 7q[83-92], and polymorphisms in the *DLG5* gene on chromosome 10q[70,93-102]. (See section C.4.1.) Haplotypes in the terminal exons of the chromosome 7p Nod1 (*CARD4*) gene and a Nod1 (*CARD4*) indel polymorphism were associated in two different study populations[47], although other studies have not replicated evidence for association[103-105]. Finally, studies of candidate genes that are not located within IBD linkage regions have revealed weak associations between UC and a variable number of tandem repeats polymorphism in the *IL1RN* gene[106] and between IBD and the TLR4 gene[107-113]. In a large case-control study, association was observed in UC and CD for Ala1011Ser within the Myo IXb (*MYO9B*) gene[114] that had been previously been implicated in celiac disease[115]. If replicated, this would indicate shared susceptibility pathways between multiple types of intestinal inflammation.

GENOME-WIDE ASSOCIATION STUDIES IN IBD

Emerging technologies now provide for the direct testing of a large number of SNPs throughout the genome in genome-wide association studies. The multiple genome-wide association studies reported below vary with respect to the genotyping platform utilized and the population cohort tested. Despite these differences, however, the first glimpses of comparative results between the genome-wide association studies are demonstrating encouraging consistency of findings between studies. Taken together, the early results from these studies would suggest that significant advances in defining well-replicated gene associations in IBD, especially in European ancestry cohorts, will shortly ensue.

TNFSF15 association to CD

The first genome-wide association study in IBD involved testing nearly 80 000 SNPs in a Japanese CD cohort[116]. The most significant findings implicated SNPs and haplotypes within the TNFSF15 (TNF superfamily) gene. These results were subsequently confirmed in European IBD cohorts[117]. TNFSF15 is a Th-1 polarizing cytokine which has been reported to be increased in IBD mucosa[118]. In a separate Belgian CD cohort, however, no significant evidence for association was observed[119]. These differences could reflect differences in markers genotyped and/or different contributions of susceptibility genes between Asian and European IBD cohorts. Future studies in a variety of population cohorts will provide important comparative insight.

IL23R is associated with CD and UC

A genome-wide association study testing over 300 000 autosomal SNPs was recently reported[120]. Three SNPs had nearly two orders of magnitude greater significance compared to the next most significant markers. Two of the three markers, were in the known CD susceptibility gene, Nod2. The third marker, rs11209026 ($P = 5.05 \times 10^{-9}$, corrected $P = 1.56 \times 10^{-3}$), was a non-synonymous SNP (Arg381Gln) in the *IL23R* gene on chromosome 1p31. Replication of these findings was observed in an independent cohort of 883 nuclear families and observed significant association in CD and non-Jewish UC. Therefore, *IL23R* represents both a CD and a UC susceptibility gene. The less common glutamine allele of Arg381Gln has an allele frequency of 1.9% in non-Jewish CD and 7.0% in non-Jewish controls and therefore protects against IBD[120]. Other variants within *IL23R* are also associated with IBD, independent of the Arg381Gln association. Since the initial report, these findings have subsequently been replicated in Scottish pediatric IBD[121] and Belgian CD[119] cohorts, indicating a clear role for *IL23R* in IBD susceptibility.

Given the IL-23 pathway's role in activation and perpetuation of organ-specific inflammatory responses, the genetic findings suggest that targeting the IL-23 pathway may be a rational therapeutic approach. In this regard, anti-p40 administration, which blocks both IL-23 and IL-12 activities, has proved promising[122]. The contribution of the *IL23R* pathway to IBD will likely involve more than simple gain or loss of function *IL23R* variants and ongoing studies of this pathway may reveal new therapeutic options. Future studies should examine mechanisms of the strong protective effect of the Arg381Gln allele could potentially be exploited to define clinical outcomes. The genetic association and the pro-inflammatory role of IL-23 strongly prioritize this pathway as a therapeutic target in IBD.

The *IL23R* genetic association to IBD correlates precisely with recent immunologic advances in understanding of the IL-23 pathway. A number of murine models of colitis, including IL-10 deficiency, T cell mediated (CD45Rbhigh reconstituted in RAG deficiency) and non-T cell mediated (agonistic anti-CD40 RAG deficiency, H. hepaticus-induced) colitis demonstrated significant amelioration when crossed with p40 and p19-deficient mice. This demonstrates the requirement for an intact IL-23 pathway for a variety of intestinal inflammation pathways[123-126].

A complete understanding of the strongly protective effect of *IL23R* polymorphisms in IBD should not be solely restricted to pharmacologic approaches to mimic the Arg381Gln polymorphism. While it is logical to test anti-p19 antibodies in IBD[127], the underlying paradigm is one of an ongoing requirement for continued immune

suppression. However, it is unlikely that the approximately 14% of European ancestry individuals heterozygous for the glutamine allele harbor significant immunodeficiencies, and therefore these individuals provide a unique opportunity to better understand the lifelong, dynamic host-intestinal microbial interactions that are the key to IBD. We are born with a sterile intestine[25] and an immature immune system[128], with the rapid acquisition of intestinal flora evolving dynamically with early instruction signals to the host immune response. The significant susceptibility of neonates to infection may correlate with their incapacity to induce IL-12p35 with lipopolysaccharide stimulation[129], with IL-23p19 induction being largely intact[130]. This would suggest that functional IL-23 pathway polymorphisms may be particularly important in modulating neonatal development of intestinal tolerance and bacterial colonization.

ATG16L association to CD

In a genome-wide survey of nearly 20 000 nonsynonymous SNPs, an amino acid polymorphisms, Thr300Ala within the *ATG16L1* gene was found to be highly associated with CD. The *ATG16L1* protein is comprised of N-terminal APG16 domain consisting of coiled coils and eight C-terminal WD repeats. The Thr300Ala variant is located at the N-terminus of the WD-repeat domain in *ATG16L1*. The *ATG16L1* gene is part of the autophagosome pathway and has been implicated in the processing of intracellular bacteria. Of interest, no association was observed in UC and a statistical interaction was reported with the Nod2 (*CARD15*) CD associations. Since the initial report, this association has been confirmed in a separate, Belgian CD cohort[119]. In addition, in this cohort, no association was observed in UC. Taken together, the *ATG16L1* association represents a well-replicated CD-specific association.

Association of a gene desert on chromosome 5p13.1 which modulates the expression of the prostaglandin receptor EP4 (*PTGER4*)

In a genome-wide association study in a Belgian CD cohort, in addition to the *CARD15*, *IL23R* and *ATG16L1* associations, association was observed in a region on chromosome 5p13.1. The most significant association was observed in a gene desert region flanked by a number of potential candidate genes, including *CARD6*, complement factors C6, C7 and C9 and the prostaglandin receptor, EP4 (*PTGER4*). No association was observed in UC for markers in this region. *PTGER4* is a compelling candidate, in part because *PTGER4* deficient mice develop a more severe colitis with dextran sodium sulfate treatment[131]. In elegant analyses, the investigators compared SNP data in this genomic region with mRNA expression levels of flanking genes from the corresponding, individual lymphoblastoid cell lines. Throughout the region of association, a number of SNPs were significantly associated with mRNA expression levels of *PTGER4*, including at least one SNP demonstrating both CD association as well as correlation with *PTGER4* expression. However, the susceptibility allele at this marker corresponds with increased *PTGER4* expression, which is not consistent with the increased colitic susceptibility observed in *PTGER4*-deficient mice[131]. However, taken together, these findings strongly suggest that genetic variation in this region is associated with IBD and significantly regulates *PTGER4* expression.

Assessment of genome-wide association studies in IBD

It is anticipated that the most significant associations observed in various genome-wide association studies will be largely replicable between studies in comparable population cohorts. Genome-wide association studies provide a comprehensive, unbiased landscape of common variation contributing to disease. Three relative limitations to these studies should be noted, however. Apart from the highly significant, outlying associations such as those observed for the Nod2 and *IL23R* gene associations, many of the most biologically significant disease associations may confer less significant statistical associations, and be obscured by surrounding "noise" conferred by false positive associations. Study design approaches to dissect true disease associations from surrounding noise remains a major methodological challenge moving forward. A second limitation is that the genotyping platforms typically utilized for these studies are limited to testing common genetic variation, with minor allele frequencies of greater than 5%. In the presence of significant allelic heterogeneity for uncommon variants within a disease gene, genome-wide association approaches will be relatively insensitive. The Nod2 associations are observed in genome-wide surveys so clearly because all three of the uncommon CD variants by chance share a common haplotype background. Therefore, the identification and characterization of uncommon variants may largely be missed by a complete reliance of genome-wide association approaches. Finally, many of the early genome-wide genotyping platforms were specifically developed to efficiently test European ancestry cohorts, and may not as efficiently assay Asian or African populations. Particularly for African populations which have shorter regions of linkage disequilibrium, testing of larger numbers of genetic markers will be required.

From genetics to genetic epidemiology: defining risks in patient subsets to assist clinical practice

A more complete pathophysiologic genetic definition of IBD will require integration of clinical observations, genetic data, statistical analyses, and delineation of underlying biologic processes. Interrogating GWA data for gene-gene interactions analyses has been proposed as being more powerful than traditional, single locus analyses under a range of interactive models. However such analyses may be more powerful if prior biologic knowledge on signaling pathways, *in vivo* models of inflammation and integration of genetic insight is applied. The growing catalogue of functional polymorphisms contributing to distinct chronic inflammatory disorders, while not necessarily demonstrating disease association, may contribute to IBD pathophysiology as disease modifiers. Stratification of genetic associations by phenotype variation and/or use of covariates may benefit genetic understanding of IBD by reducing heterogeneity that can obscure association evidence. Because of the strength of the Nod2 association

in CD, and because ileal CD represents a large fraction of overall cases of CD, the Nod2 associations were easily observed in the "all CD" analyses, and even the "all IBD" analyses. However, as the Nod2 genotype risk represents one of the largest among complex disorders, it would be anticipated that many IBD association signals may require correct subsetting, of which disease location is most well-established. Integration of clinical and genotype data may provide the capacity to predict complications and disease course in a clinically useful way. While the integration of definitive IBD risk alleles would be the most straightforward factors to include in such predictive models, it is possible that many key genetic factors (particularly well-established functional polymorphisms from related disorders) may, by themselves, not be associated with the major phenotypes, but rather function solely as disease modifiers. For example, it is possible that certain key genetic factors (particularly well-established functional polymorphisms from related disorders) may, by themselves, not be associated with the major phenotypes but rather serve to modify disease expression. Detecting such relationships requires a conditional analysis restricted to those with IBD in which certain phenotypic characteristics are modeled as a function of the presence of one or more genetic variants. Such models will also provide a framework for generating predictions about clinical course among newly diagnosed cases-providing clinicians important information for use in treatment planning.

The identification of major single gene associations such as *IL23R* and Nod2 provides a framework around which risk models for multigenic disorders can be developed. A major research interest moving forward will be identifying gene-gene interactions that contribute to increased disease risk or define pathophysiological subsets within the disease. This will be explored through: (1) a broad-based, systematic search of genomic regions demonstrating the most significant evidence for association, (2) pathway analyses integrating specific knowledge regarding functional human polymorphisms relevant to the *IL23R* and Nod2 pathways, and (3) studying key mechanistic intermediates known to be particularly relevant to disease pathogenesis.

As functional genetic variants that predispose to IBD are identified, their effect on modifying disease phenotype and/or disease course will need to be examined. For example, the CARD15 mutations increase susceptibility to CD generally, affect disease location (increase susceptibility to ileal location), and modify disease behavior. Because of the challenges of longitudinal follow-up, prospective evaluation of IBD-associated mutations represents a major challenge. The efficient abstraction of critical research datapoints from active clinical practices in an accurate and reproducible manner will be required. Interpretation of disease course from multi-center studies given differences in practice patterns represents an additional interpretative challenge. However, such studies will provide the basis for improved translation of genetic discovery in IBD and will need to be designed *via* active communication and collaboration throughout the IBD research community.

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TOPIC HIGHLIGHT

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Epithelial restitution and wound healing in inflammatory bowel disease

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Abstract

Inflammatory bowel disease is characterized by a chronic inflammation of the intestinal mucosa. The mucosal epithelium of the alimentary tract constitutes a key element of the mucosal barrier to a broad spectrum of deleterious substances present within the intestinal lumen including bacterial microorganisms, various dietary factors, gastrointestinal secretory products and drugs. In addition, this mucosal barrier can be disturbed in the course of various intestinal disorders including inflammatory bowel diseases. Fortunately, the integrity of the gastrointestinal surface epithelium is rapidly reestablished even after extensive destruction. Rapid resealing of the epithelial barrier following injuries is accomplished by a process termed epithelial restitution, followed by more delayed mechanisms of epithelial wound healing including increased epithelial cell proliferation and epithelial cell differentiation. Restitution of the intestinal surface epithelium is modulated by a range of highly divergent factors among them a broad spectrum of structurally distinct regulatory peptides, variously described as growth factors or cytokines. Several regulatory peptide factors act from the basolateral site of the epithelial surface and enhance epithelial cell restitution through TGF-β-dependent pathways. In contrast, members of the trefoil factor family (TFF peptides) appear to stimulate epithelial restitution in conjunction with mucin glycoproteins through a TGF-β-independent mechanism from the apical site of the intestinal epithelium. In addition, a number of other peptide molecules like extracellular matrix factors and blood clotting factors and also non-peptide molecules including phospholipids, short-chain fatty acids (SCFA), adenine nucleotides, trace elements and pharmacological agents modulate intestinal epithelial repair mechanisms. Repeated damage and injury of the intestinal surface are key features of various intestinal disorders including inflammatory bowel diseases and require constant repair of the epithelium. Enhancement of intestinal repair mechanisms by regulatory peptides or other modulatory factors may provide future approaches for the treatment of diseases that are characterized by injuries of the epithelial surface.

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THE MUCOSAL DEFENSE SYSTEM

The surface of the digestive tract is covered by epithelial cells that constitute an efficient physical barrier between the dietary and enteric flora pathogens found in the intestinal lumen and the individuum, but also allows an exchange between nutrients and the systemic circulation[1]. The epithelial defense mechanism can be categorized into three key components: pre-epithelial, epithelial and post-epithelial, the latter is represented by the lamina propria[2]. The pre-epithelial mucus barrier is composed of mucin associated with other proteins and lipids and forms a continuous gel into which a bicarbonate-rich fluid is secreted, maintaining a neutralizing pH at the epithelial surface. Phosphatidylcholine is the predominant surface bioactive phospholipid found within the gastrointestinal tract[3]. Intestinal epithelial cells secrete mucins and glycocalyx, which contain membrane-anchored negatively charged mucin-like glycoproteins and hydrophobic phospholipids[4]. The tight adherence of mucin to the apical surfaces of epithelia is owed to the existence of the specific complex between mucin oligosaccharides and the mucin binding protein of the apical mucosal membrane[5]. The hydrophobic lining of the luminal surface has an important functional role. It prevents microorganisms

Table 1 Important modulators of intestinal epithelial cell function

Localization	Modulator
Gastrointestinal lumen	Dietary compounds
	Alimentary secretions from salivary glands, stomach, pancreas or intestinal glandular cells
	Secreted regulatory peptides
	Physiological and pathogenic intestinal microflora
	Non-peptides factors (e.g. phospholipids, polyamines, short chain fatty acids and other)
	Drugs
	Other
Epithelium	Regulatory peptides
	IEL
	Local cell-cell interactions
	Non-peptide factors (e.g. phospholipids, adenine nucleotides, polyamines and others)
	Other
Lamina propria and basal lamina	Regulatory peptides expressed by various constituents of the lamina propria
	Extracellular matrix factors
	Neurotransmitters
	Nerval interactions
	Various mediators that are transported *via* the blood stream
	Non-peptide factors (e.g. phospholipids, adenine nucleotides, polyamines and others)
	Other

from getting into contact with and to adhering to the plasma membrane. It furthermore protects the mucosal epithelium against chemical and mechanical injuries[6]. Epithelial cells provide the second line of the mucosal defense system. Whereas in the upper digestive tract this layer consists of a stratified epithelium, the stomach, small, and large bowel are surfaced with a simple epithelial layer sealed by tight junctions[7]. When intact, the uptake of antigens, macro- and microorganism through this layer is restricted by luminal cell-surface structures. The mucosal surface epithelial cells are rapidly proliferating with a complete turnover every 24 to 96 h[8]. The proliferative compartment of epithelial cells is localized in the crypt region and is segregated from a gradient of increasingly differentiated epithelial cells present along the vertical axis of the functional villus compartment[9,10].

INTESTINAL WOUND HEALING

Damage and impairment of the intestinal surface barrier are observed in the course of various diseases and may result in an increased penetration and absorption of toxic and immunogenic factors into the body leading to inflammation, uncontrolled immune response, and disequilibrium of the homeostasis of the host. Thus, rapid resealing of the epithelial surface barrier following injuries or physiological damage is essential to preserve the normal homeostasis. Observations over the past several years have demonstrated the ability of the intestinal tract to rapidly reestablish the continuity of the surface epithelium after extensive destruction[11-14]. The continuity of the epithelial surface is reestablished by at least three distinct mechanisms. First, epithelial cells adjacent to the injured surface migrate into the wound to cover the denuded area. Those epithelial cells that migrate into the wound defect dedifferentiate, form pseudopodia-like structures, reorganize their cytoskeleton, and redifferentiate after closure of the wound defect. This process has been termed epithelial restitution and does not require cell proliferation[15]. Intestinal epithelial restitution occurs within minutes to hours both *in vivo* and *in vitro*. Secondly, epithelial cell proliferation is necessary to replenish the decreased cell pool. Third, maturation and differentiation of undifferentiated epithelial cells is needed to maintain the numerous functional activities of the mucosal epithelium. The separation of intestinal epithelial wound healing in three distinct processes is rather artificial and simplified. These three wound-healing processes overlap and distinct processes may not be observed *in vivo* where these processes overlap. The preservation of this barrier following injuries is regulated by a broad spectrum of structurally distinct regulatory factors, including cytokines, growth factors, adhesion molecules, neuropeptides and phospholipids[16-19]. However, this artificial and simplified model provides a tool to better understand the physiology and pathophysiology of intestinal epithelial wound healing. Moreover, deeper lesions or penetrating injuries will require additional repair mechanisms that involve inflammatory processes and non-epithelial cell populations. Inflammatory processes especially may interfere with epithelial cell migration and proliferation and thus modulate intestinal epithelial healing.

IMPORTANT MODULATORS OF THE INTESTINAL EPITHELIAL CELL FUNCTION

The epithelial cell populations of the intestinal mucosa are modulated by a number of factors that are present within the lumen, the epithelium itself or the underlying lamina propria (Table 1). Although the full variety of regulatory factors that play a role in the control of intestinal epithelial and non-epithelial cell populations has not been fully defined yet, there is increasing appreciation of the diversity of these factors in general and the importance of several specific peptide and non-peptide factors produced or released within the intestine. The identification and characterization of numerous regulatory peptide and non-peptide factors has led to the recognition of a network of interrelated factors within the intestine (Figure 1). The constituents of this network generally possess multiple functional properties and exhibit pleiotropism in their cellular sources and targets. As a result, this network is highly redundant in several dimensions[20]. Regulatory peptides especially seem to play a key role in intestinal epithelial wound repair, as they are abundantly detectable in the intestinal lumen, intestinal epithelium and the underlying lamina propria. Various members of several distinct regulatory peptide families have been recognized to modulate a broad spectrum of intestinal epithelial cell functions including cell migration, proliferation and/or differentiation (Figure 2). As outlined above the latter epithelial cell functions are highly relevant for the modulation of intestinal epithelial wound repair.

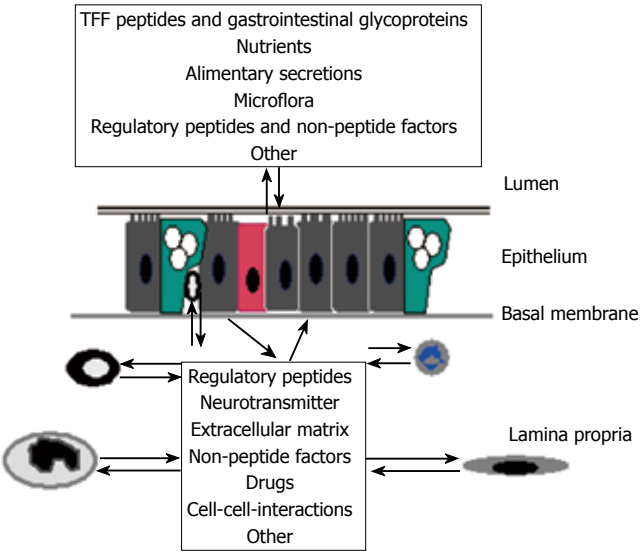


Figure 1 Regulatory network within the intestinal mucosa (adopted and modified from Reference 32).

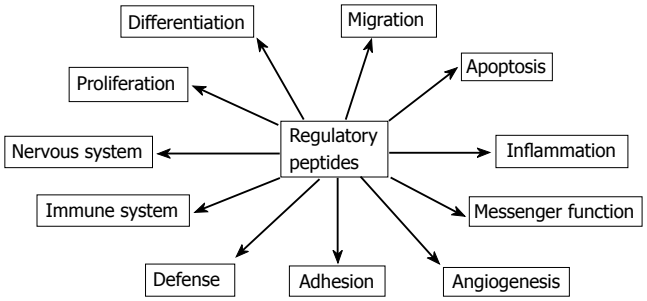


Figure 2 Functional activities of peptide growth factors within the intestinal mucosa (adopted and modified from Reference 32).

THE ROLE OF REGULATORY PEPTIDES FOR INTESTINAL EPITHELIAL WOUND HEALING

A broad spectrum of structurally distinct regulatory peptides is expressed from various cell populations within the mucosa of the intestinal tract. These regulatory peptides, conventionally designated as growth factors and cytokines play an essential role in regulating differential epithelial cell functions in order to preserve normal homeostasis and integrity of the intestinal mucosa[20-25]. The terminology of regulatory peptides is often confusing and arbitrary. The term cytokines is now increasingly used to describe a bunch of regulatory peptides that can be variously identified as regulatory peptides, peptide growth factors, interleukins, interferons and colony stimulating or hematopoetic stem cell factors. For the purpose of simplification, the term regulatory peptide or cytokine will be used in this paper to address all the different classes of regulatory peptide factors. Regulatory peptides can be reasonably classified on the basis of structural homologies and disparities into several discrete families. Peptide growth factor families and selected members with functional activities in the modulation of intestinal wound healing. In addition to growth factor families, a number of regulatory peptide factors, seemingly without structural similarities to other regulatory peptide family members like vascular endothelial cell growth factor (VEGF) and platelet-derived growth factor (PDGF) have been identified to be expressed within the intestinal tract and to modulate wound healing properties within the intestinal mucosa[25]. Furthermore, a countless number of classical cytokines like IL-1, IL-2, IL-15, IL-22 and IFN-γ are expressed within the intestine and modulate numerous intestinal epithelial cell functions[26-30].

The various effects of a number of regulatory peptides on cell adhesion, migration, proliferation, differentiation, intestinal epithelial barrier function and angiogenesis suggest that these peptides are likely relevant factors for intestinal repair mechanisms. Both *in-vitro* and *in-vivo* studies have demonstrated that several growth factors and cytokines can enhance epithelial cell restitution[31,32]. A spectrum of growth factors and cytokines including EGF, VEGF, HGF, GLP-2, various FGF peptides, IL-1, IL-2 and IFN-γ have been demonstrated to enhance epithelial cell restitution through a TGF-β-dependent pathway[33-36]. Interestingly, it appears that restitution-enhancing cytokines use different mechanisms to modulate TGF-β-peptide levels. While TGF-β, EGF, IL-1, IFN-γ and HGF only increased the concentration of bioactive TGF-β, acidic and basic FGF and also IL-2 enhanced both the bioactivation of TGF-β and the expression of TGF-β mRNA and production of latent TGF-β peptide. This might reflect a different mechanism, by which acidic and basic FGF and also IL-2 modulate the synthesis and bioactivation of TGF-β. In contrast to the above mentioned growth factors and cytokines that are assumed to act from the basolateral site of the epithelial surface and that seem to stimulate intestinal epithelial restitution through a common TGF-β-dependent pathway, various members of the trefoil factor family (TFF Peptide family) appear to stimulate epithelial restitution in conjunction with mucin glycoproteins through a TGF-β-independent mechanism from the apical pole of the epithelium[35,37,38]. In this regard, our group recently also demonstrated that mesalamine promotes intestinal wound healing *in vitro* through a TGF-β-independent mechanism[39]. Recent studies suggest that modulation of repair mechanisms by trefoil peptides may be mediated by modulation of the E-cadherin/catenin complex[40,41]. However, a double-blind, randomized, placebo-controlled study treating 16 patients with mild-to-moderate left sided ulcerative colitis with enemas containing human recombinant trefoil factor family-3 did not reveal any additional benefit above that of adding 5-aminosalicylic acid alone[42].

It has been demonstrated, that keratinocyte growth factor (KGF) has an important function in wound re-epithelialization[43] and KGF expression is strikingly increased in surgical specimens from patients suffering from Crohn's disease and ulcerative colitis[44]. Furthermore, in an experimental model of colitis, administration of KGF after but not before induction of colitis significantly ameliorated tissue damage demonstrating that exogenous KGF might promote IBD[45]. However, clinical studies

Table 2 Selected non-peptide factors with relevance for intestinal epithelial wound healing

Factor	Mechanism of action	References
Lysophosphatidic acid	Stimulates intestinal epithelial restitution and inhibits intestinal epithelial proliferation	[61,67]
Polyamines	Stimulate intestinal epithelial restitution and proliferation	[11,66,75]
Adenine nucleotides	Stimulate intestinal epithelial restitution and inhibit intestinal epithelial proliferation	[59]
Short chain fatty acids	Stimulate intestinal epithelial migration	[63,76]
Glutamine	Stimulates intestinal epithelial proliferation and migration	[76,77]

treating patients with CD or UC with exogenous KGF have not been performed yet. In addition to their potent effects on epithelial restitution, a number of regulatory peptide factors act also as potent modulators of epithelial cell proliferation[17,18,32,46-49]. The most important modulators of intestinal epithelial cell proliferation include EGF and TGF-β which both act as potent stimulators of intestinal epithelial proliferation and TGF-β which inhibits intestinal epithelial cell proliferation and plays an important counterbalancing role in the regulation of intestinal epithelial cell proliferation. TGF-β is the most potent inhibitor of intestinal epithelial cell proliferation overriding the stimulatory effects of other stimulatory factors. The growth stimulating effect of IL-2, FGF peptides, IGF and HGF is rather moderate compared to the effects of EGF and TGF-β that stimulate epithelial cell proliferation five- to ten-fold in several intestinal epithelial cell lines *in vitro*[33,34,48,50]. Thus, it is not astonishing that in a randomized, double-blind clinical trial, after a 2-wk treatment period, patients receiving EGF enemas had a significant lower disease activity score than the control patients[51]. Concerning basic FGF, in a mouse DSS-model of experimental colitis, rectal administration of human recombinant basic FGF ameliorated the inflammation score and suppressed TNF-α gene expression in the colonic tissue[52,53]. Recently, the important role of Glucagon-like-peptide-2 (GLP-2), which is secreted from local neuroendocrine epithelial cells and promotes epithelial cell proliferation *via* stimulation of enteric neurons[54], has received more attention. GLP-2 demonstrating ability to ameliorate murine short bowel syndrome and experimental colitis[55,56] has consequently lead to clinical studies, evaluating the effect of GLP-2 in patients with short bowel syndrome.

However, it has also to be considered that cytokines have pleiotropic activities. For example, FGF also induces stricture formation in Crohn's disease[57] and GLP-2 might have undesired effects in tumorogenesis[58], which might limit their therapeutic use. In addition, it has to be considered that these factors and also various non-peptide factors may act in an additive or even synergistic fashion which may potentiate their single effects. Notably, in addition to TGF-β the TGF-β family member Activin A has been identified to also inhibit epithelial cell proliferation, thus providing an additional mechanism to counterbalance the effects of proliferative factors present within the intestinal mucosa and to inhibit unrestrained cell growth[59,60].

MODULATION OF INTESTINAL EPITHELIAL WOUND HEALING BY NON-PEPTIDE FACTORS

In addition to the potent modulation of intestinal epithelial wound healing by regulatory peptides, it is increasingly appreciated that a broad spectrum of non-peptide factors exerts potent effects on intestinal epithelial cell populations and modulates those epithelial cell functions that are involved in the healing of intestinal injury (Table 2). These non-peptide factors encompass a broad spectrum of unrelated factors like phospholipids, nutrients (adenine) nucleotides, polyamines, short chain fatty acids (SCFA), products of the intestinal microflora, trace elements, pharmacological agents and other factors. Some of these factors are released by injured or dying mucosal cell populations (e.g. adenine nucleotides, phospholipids), other reach the intestinal mucosa *via* the intestinal lumen or the blood stream. These non peptide factors may exert growth factor like activities and exert potent effects on cell growth and differentiation in different cell populations including fibroblasts, vascular smooth muscle cells, endothelial cells and keratinocytes[20,35,61-63]. As some of these non-peptide factors are stable within the gastrointestinal tract despite high concentrations of acid, bile salts, proteases and microorganisms and as they exhibit only limited toxicity *in vivo*, they may serve as potential future targets to improve the armamentarium for the healing of mucosal epithelial injury.

Especially phospholipids and polyamines seem to be of special interest, as these non-peptide factors can be easily added to the regular diet and their overall content and biological activity can be modulated by various pharmacological agents[62,64-66]. Lysophosphatidic acid (LPA) is a key intermediate in the early steps of phospholipid biosynthesis and is rapidly produced and released from thrombin-activated platelets and growth factor stimulated fibroblasts to influence target cells by activating a specific 38-40 kDa G-protein coupled receptor that is expressed in many cells[67]. As a product of the blood-clotting process, LPA is a normal constituent of serum, where it is present in an albumin-bound form in physiologically relevant concentrations[64]. Major sources of LPA in the vicinity of injured epithelial cells are activated platelets, stimulated fibroblasts and presumably injured cells that release LPA due to non-specific phospholipase activation[67]. LPA promotes platelet aggregation and induces cellular tension and cell surface fibronectin assembly[68], which are also important events in wound repair suggesting an important role of LPA in inflammatory disorders. This was confirmed by our group when we demonstrated, that LPA not only promotes epithelial wound healing *in vitro* by a TGF-β-independent pathway, but also ameliorates experimental colitis in an experimental model of colitis in rats[61]. Interestingly, also lysophosphatidylethanolamine and lisofylline, which decreases lipid peroxidation, significantly

reduced the degree of inflammation and necrosis in an experimental colitis model[69], demonstrating that the administration of anti-inflammatory lysophospholipids and suppression of pro-inflammatory lipid metabolites by lisofylline may provide new approaches to ameliorate intestinal inflammation. This beneficial effect could also be demonstrated in other diseases since lisofylline and its analogs reversed autoimmune diabetes in a non-obese diabetic (NOD) mouse model and thus might act as a potential treatment for Type 1 diabetes[70,71].

Many studies have reported that bone marrow cells may have the potential to contribute to the repair of many non-hematopoetic tissues, including the intestinal epithelial cells. Bone-marrow derived cells are capable of promoting regeneration of damaged intestinal epithelial cells[72]. However, the underlying effects are not fully understood[18]. Nevertheless, bone-marrow transplantation combined with immune-suppressive therapy improves epithelial wound healing[73] and recombinant granulocyte-macrophage colony-stimulating factor have a therapeutic effect in patients with active Crohn's disease[74].

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TOPIC HIGHLIGHT

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Treatment of inflammatory bowel disease: A review of medical therapy

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Abstract

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory diseases of the gastrointestinal tract. While a cure remains elusive, both can be treated with medications that induce and maintain remission. With the recent advent of therapies that inhibit tumor necrosis factor (TNF) alpha the overlap in medical therapies for UC and CD has become greater. Although 5-ASA agents have been a mainstay in the treatment of both CD and UC, the data for their efficacy in patients with CD, particularly as maintenance therapy, are equivocal. Antibiotics may have a limited role in the treatment of colonic CD. Steroids continue to be the first choice to treat active disease not responsive to other more conservative therapy; non-systemic steroids such as oral and rectal budesonide for ileal and right-sided CD and distal UC respectively are also effective in mild-moderate disease. 6-mercaptopurine (6-MP) and its prodrug azathioprine are steroid-sparing immunomodulators effective in the maintenance of remission of both CD and UC, while methotrexate may be used in both induction and maintenance of CD. Infliximab and adalimumab are anti-TNF agents approved in the US and Europe for the treatment of Crohn's disease, and infliximab is also approved for the treatment of UC.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Medical therapy

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are the principle syndromes encompassed by the classification of inflammatory bowel disease (IBD). While CD can affect any part of the gastrointestinal tract, it most commonly occurs in the distal ileum and colon, whereas UC by definition affects only the colon. The etiology appears multifactorial: an underlying immune dysregulation coupled with an intolerance to gut flora seems fundamental to the pathogenesis that, in some cases, are associated with genetic mutations or are initiated by environmental factors. Apart from a total proctocolectomy for UC, there is no cure for IBD. Medications, however, aid in the induction and maintenance of remission, and target various points along the disordered immune pathway implicated in IBD.

CROHN'S DISEASE

Aminosalicylates

While there is solid data supporting 5-aminosalicylic acid (5-ASA, mesalazine or mesalamine) in the induction and maintenance of remission for UC, their efficacy in the treatment of CD is not as clear. Interpretation of the data is often confounded by the use of different formulations, doses, and varied applications to different disease scenarios (disease location, concomitant medications, and prior therapies). 5-ASA agents are likely to have multiple anti-inflammatory effects, including inhibition of cyclooxygenase, lipoxygenase, B-cells, and several key inflammatory cytokines. Most recently, 5-ASA has been shown to activate selective peroxisome proliferators-activated receptor ligand-γ (PPAR-γ), a nuclear receptor that controls cell proliferation and apoptosis[1]. Originally designed as treatment for rheumatoid arthritis (RA), sulfasalazine was discovered to also benefit patients with IBD[2]. An azo-bond links sulfapyridine to 5-ASA and is cleaved by bacterial azo-reductase in the colon, thus allowing delivery of the active 5-ASA moiety to

the large intestine. Limitations of sulfasalazine include allergic reactions and side effects, largely attributed to the sulfapyridine moiety, as well as its lack of efficacy in isolated small bowel disease that is proximal to the colonic release of 5-ASA. Two newer non-sulfa-containing 5-ASA agents, balsalazide and olsalazine were developed to treat colonic inflammation; further, mesalamine formulated to release in a pH (Asacol®, Claversal®, Mesasal®, and Salofalk®) or time-dependent manner (Pentasa®) can treat either small or large bowel CD[2].

Active disease

The National Cooperative Crohn's Disease Study (NCCDS) and European Cooperative Crohn's Disease Study (ECCDS) were large multicenter randomized controlled trials published in 1979 and 1984 that evaluated the comparative efficacy of sulfasalazine, prednisone and azathioprine in the treatment of both active and quiescent CD. While the NCCDS found sulfasalazine 6 g/d superior to placebo overall in treating active disease, when stratified by disease location only those with colonic and ileocolonic (but not isolated small bowel) disease obtained benefit[3]. In contrast, the ECCDS did not demonstrate efficacy for sulfasalazine 3 g/d alone, but only in combination with 6-methylprednisolone[4].

Subsequently, newer mesalamine agents have been evaluated in clinical trials for CD. In the largest of these studies (n = 310), patients with active ileal or ileocolonic CD were randomized to receive Pentasa®, 1, 2 or 4 g/d or placebo. The 4 g/d group experienced a greater decrease in CDAI than the placebo group (72 *vs* 21 points, $P < 0.01$), an effect more pronounced in isolated ileal disease, and remission was achieved in 43% *vs* 18% respectively[5]. Subsequently, two similarly designed trials described in a meta-analysis failed to replicate these findings although there was an overall statistical benefit for the 4 g dose of mesalamine that was of questionable clinical significance[6,7]. Several other trials also have demonstrated benefit for mesalamine in CD, but the quality of the trials was less robust[8,9]. When compared to other agents in controlled trials approximately 40%-55% of patients treated with mesalamine 4 g/d achieve remissions but the efficacy was less than budesonide (9 mg/d) for the induction of remission at both 8 wk (45% *vs* 65%, $P = 0.001$) and 16 wk (36% *vs* 62%, $P < 0.001$)[10] and comparable to ciprofloxacin 1 g/d[11].

Maintenance after medical remission

Sulfasalazine at reduced doses compared to the induction phase provided no benefit compared to placebo in the maintenance phase of the NCCDS and ECCDS, nor in a smaller study[3,4,12]. Gisbert *et al* have reviewed nine randomized placebo-controlled studies of mesalamine as a maintenance agent, four of which showed a significantly decreased risk of relapse compared to placebo, although there was great heterogeneity in formulation, dosage, duration of treatment, and disease location[13]. Further, a Cochrane review of seven randomized placebo-controlled trials concluded that treatment with 5-ASA agents for at least six months did not confer an advantage over placebo in patients with medically-induced remission[14]. When initiated within three months of a medically-induced remission, mesalamine (2 g bid), in contrast to placebo, prevented more relapses over a two year period[15]. In the context of a steroid-induced remission, short-term weaning from steroids may be slightly facilitated with mesalamine 4 g/d, but there was no benefit at one year in relapse rate between patients maintained on mesalamine compared with placebo[16].

Post-operative maintenance

The natural history of CD after ileocolonic resection is variable, and may be influenced by such factors as pattern, extent, and duration of disease pre-operatively as well as smoking history. Endoscopic recurrence rates range from 28%-93% at one year[17], while clinical relapse rates have been reported at 20% and 34% at one and three years respectively[18]. Approximately 30% of patients require re-operation within 10 years[17], highlighting the relevance of identifying an effective post-operative maintenance strategy. Except for one study that showed a benefit at one (but not three) years, sulfasalazine has not been statistically superior to placebo in preventing post-operative relapse[17]. Data for mesalamine has been equivocal in the setting of post-operative maintenance trials. While a meta-analyses of 15 randomized controlled studies (n = 2097) of mesalamine as a maintenance medication in CD found a 13% pooled risk reduction for those patients with surgically-induced remissions[19], the largest (n = 318) and most rigorously conducted trial to date in which patients began mesalamine therapy (4 g/d) within ten days of surgery did not show benefit over placebo. While a post-hoc analysis did suggest efficacy for patients with isolated small bowel disease (21% *vs* 39% relapse rate, $P < 0.02$)[20], if this trial had been included in the meta-analysis, the overall findings of benefit compared to placebo would no longer have been significant[21]. Most recently, mesalamine at 3 g/d was inferior to mercaptopurine at 50 mg/d at preventing post-operative recurrence[22].

In summary, for the treatment of mild to moderate active CD, 5-ASA agents, while less efficacious than budesonide for ileal and or right colonic disease, may be a reasonable choice as first-line therapy: sulfasalazine should be reserved for patients with predominantly colonic disease, while time or pH-dependent release mesalamine are appropriate for patients with small bowel disease. The role of 5-ASA as a maintenance medication is equivocal at best, but is clearly of no benefit in patients with a steroid-induced remission and in the setting of post-operative maintenance, at least 3 g/d would need to be initiated immediately after surgery to provide any benefit for patients with small bowel disease.

ANTIBIOTICS

Active disease

Metronidazole, ciprofloxacin, combination anti-mycobacterials, and most recently ornidazole and rifaximin have been evaluated in the treatment of active CD. The few randomized controlled trials to study the efficacy of metronidazole and/or ciprofloxacin have been mostly small and provided negative results[7] despite subgroup

analyses suggesting a trend towards significant benefit in patients with colonic disease[23-25]. One small study (n = 47) showed ciprofloxacin 1 g/d for six months decreased CDAI scores significantly more than placebo ($P < 0.001$)[26] and while an eight month cross-over study between sulfasalazine 3 g/d and metronidazole 800 mg/d showed no treatment differences in the initial four months, 15 patients who switched from sulfasalazine to metronidazole had significant decreases in the CDAI compared to none of the group who crossed-over from metronidazole to sulfasalazine[27]. As previously described, 16-wk remission rates were similar for ciprofloxacin and mesalamine in a small, randomized trial[11] while another trial reported no differences in remission rates between the combination of ciprofloxacin and metronidazole *versus* methylprednisolone, despite a trend favoring steroids[28]. In contrast, a combination of ciprofloxacin and metronidazole provided no additional benefit over budesonide, alone, aside from a post-hoc analysis for patients with colonic disease[25]. A recent study that compared rifaximin 800 mg bid, 800 mg/placebo and placebo bid failed to show a significant difference between the three groups in clinical response or remission, despite a trend toward benefit with the higher dose[29].

Perianal disease and post-operative maintenance

Although antibiotic therapy is frequently used in the treatment of perianal fistulae, there are no randomized controlled trials to support this practice. Data from several small open-label trials conducted in the early 1980s reported the efficacy of metronidazole in healing perianal fistulae[30-32]. In the post-operative setting a three month course of metronidazole (20 mg/kg per day) decreased the severity of endoscopic lesions at one year (but not at two years) and delayed onset of clinical recurrence[33]. Most recently, ornidazole (1 g/d), started within 10 d of resection and continued for one year, showed significant benefit over placebo in both clinical and endoscopic recurrence rates[34]. The main limitation of long-term metronidazole and ornidazole is peripheral neuropathy.

In summary, while antibiotics are used frequently to treat perianal disease, their role in the treatment of active luminal disease and a safe and effective dose schedule in the post-operative setting, remain to be established.

SYSTEMIC STEROIDS

Mechanism of action

By binding to intracytoplasmic glucocorticoid receptors found in most cell types, glucocorticosteroids activate glucocorticoid-responsive elements (GREs), resulting in a broad spectrum of effects on the immune system including inhibition of the recruitment and proliferation of lymphocytes, monocytes and macrophages, migration of neutrophils to sites of inflammation, and decreased production of soluble inflammatory mediators including cytokines, leukotrienes, and prostaglandins[35].

Natural history

The natural history of 171 CD patients diagnosed between 1970 and 1993 has been studied in the Olmsted County, Minnesota population[36]. Of this cohort, only 43% ever required steroids before 1997 and of these, 58% were in complete remission after one month while 26% were in partial remission and 16% had no response. Of those who responded, the one-year outcomes were concerning as only 32% of patients had a prolonged response to corticosteroids, 28% became steroid-dependent and 38% had undergone surgery. These are data that are similar to the reported Danish, Copenhagen County experience[37]. Both exemplify the likelihood of developing steroid refractory or dependent disease with an accelerated course toward surgery. Hence, the requisite for steroids may be considered the "tipping point"[38] of CD that heralds a more complex subsequent course[39], including the need for surgery or the addition of an immunomodulatory agent.

Efficacy

Glucocorticosteroids are effective inductive agents for CD. The first definitive data came from the NCCDS, in which 60% of patients treated with prednisone (0.25-0.75 mg/kg per day) were in remission at 17 wk compared to 30% of placebo-treated patients[3]. Even more impressive were the results from the ECCDS in which 80% of patients treated with methylprednisolone (48 mg) achieved remission at 18 wk compared to less than 40% of placebo patients[4]. More recent randomized controlled studies have compared prednisolone (40 mg) or 6-methylprednisolone (48 mg) to budesonide (9 mg) in the treatment of active CD ileocolitis, with similar rates found for the induction of remission at 66% and 73% for the two systemic steroids[40,41].

Although in one retrospective review, 60% patients treated with alternate-day prednisone treatment (mean dose of 25 mg q.o.d.) maintained "favorable responses" for an average of 6.6 years[42], the overwhelming evidence does not support the use of corticosteroids for maintenance of remission. Neither the NCCDS nor ECCDS studies showed benefit of corticosteroids over placebo in maintaining remissions[3,4]. Conventional corticosteroids are not effective at preventing post-operative relapse[43] and a recent Cochrane review of three randomized double-blind placebo controlled studies showed no benefit of corticosteroid therapy in preventing relapses in patients with quiescent CD over 24 mo[44].

NON-SYSTEMIC STEROIDS

Budesonide, in delayed or controlled-release formulations that deliver the potent glucocorticoid to the ileum and/or right colon, has low systemic side effects owing to a high (80%-90%) first-pass metabolism[45]. Two randomized controlled studies demonstrated superiority of budesonide in the induction of remission in patients with ileal or ileo-right colonic disease[46]. In the first trial, 258 patients received 15, 9, or 3 mg of budesonide, daily, or placebo, with 43%, 51%, 33% and 20% of patients respectively achieving clinical remission in 8 wk ($P < 0.001$, $P = 0.009$ for the higher doses compared to placebo respectively)[47]. In the second study (n = 200), 9 mg/d, 4.5 mg BID twice daily budesonide or placebo yielded remission rates of 48%, 53%, and 33% respectively after 8 wk of treatment.

Although differences between the groups were not significant, when data from the two treatment groups were pooled, the budesonide group had a significantly greater decrease in CDAI than the placebo group ($P < 0.05$)[48]. One study comparing daily 18 mg, 9 mg, and 6 mg of budesonide found a dose-dependent effect, with 66%, 55% and 36% achieving remission. While for most patients, 9 mg/d is a sufficient dose, high disease activity (CDAI $\geqslant$ 300) or disease distal to the transverse colon responded better to the highest budesonide dose[49] and as discussed above, budesonide 9 mg/d has also been shown to be a more effective treatment than mesalamine for the induction of remission in mild-moderate active ileal and right-sided colonic CD[10]. When compared to prednisone, budesonide 9 mg/d there were no significant differences found in clinical remission rates[40,41,50,51] although a meta-analysis revealed the pooled rate difference of response of budesonide *vs* conventional corticosteroids to be - 8.5%, $P = 0.02$[52]. Budesonide was associated with fewer steroid side effects overall in three studies[40,41,50] and reduced incidence of moon facies and adrenal impairment in the other[51].

While extended treatment with budesonide has been shown to prolong the time to relapse compared to placebo, the difference was not sustained at one year with 3 mg[53] or 6 mg[54-56]. Similarly, another study found no difference in relapse rate at any time point over a one year period between patients treated with either 3 mg or 6 mg budesonide and placebo[57]. Neither budesonide 3 nor 6 mg/d was shown to be more effective than placebo in preventing post-operative clinical[58] or endoscopic recurrence[58,59]. Both a Cochrane review and meta-analysis confirmed that budesonide is ineffective at maintaining CD remissions[52,60]. However, in a trial that allowed flexible dosing of budesonide or prednisone over two years to maintain clinical quiescence and examined bone mineral density (BMD) in relation to efficacy and side effects in CD patients, only 37% of budesonide-treated patients withdrew from the study because of failure to improve or worsening disease. However, the average dose of budesonide required to maintain remissions was higher than (6.8 mg/d) doses used in the placebo-controlled trials. Nevertheless, among patients who were steroid-naïve prior to entering the study, smaller reductions in BMD were seen in the budesonide group compared to the prednisolone group (mean, -1.04% *vs* -3.84%; $P = 0.0084$)[61].

Budesonide at doses below 6 mg/d has been demonstrated to be safe for long-term (one year) use. Results from a pooled analysis of five one-year controlled trials using budesonide 6 mg/d showed that while the overall number of adverse events were not different between the budesonide and placebo groups, patients treated with budesonide had more endocrine and "resistance mechanism" disorders (infection) ($P = 0.0042$ and $P = 0.042$, respectively). The higher incidence of endocrine problems was primarily driven by acne and moon facies, while viral infections accounted for the difference in infection rate. Serious adverse events were reported as rare[62].

In summary, while budesonide is an effective and safe medication for the induction of remission in patients with mild-moderate ileal and proximal colonic disease, optimal dosing schedules to maintain remissions have yet to be established. While budesonide > 6 mg/d or an adjustable dose may maintain remission, a randomized controlled trial is needed to confirm the results of the open-label studies.

IMMUNOMODULATORS

Azathioprine (AZA)/6-Mercaptopurine (6-MP)

6-MP and its prodrug AZA are purine analogs that are converted into 6-thioguanine nucleotides (6-TG); the therapeutically active metabolites interfere with nucleic acid synthesis, exhibit anti-proliferative effects on activated lymphocytes and, most recently, have been shown to induce apoptosis[63,64]. These agents have been studied for the treatment of CD since the late 1960s, with multiple uncontrolled trials showing favorable results. A meta-analysis of AZA and 6-MP for the induction of remission included eight randomized placebo controlled trials ($n = 425$) while another for maintenance of remission included five trials ($n = 319$); three trials with induction and maintenance arms were included in both analyses[65,66]. For active disease, the overall response rate was 54% for patients receiving treatment compared to 33% for those on placebo, yielding a pooled odds ratio (OR) of 2.36 and the number needed to treat (NNT) for one patient to respond was 5; for quiescent disease, overall remission was seen in 67% of patients on treatment compared to 52% of those on placebo, for an OR of 2.16 and NNT of 7. In active disease, those receiving AZA or 6-MP for $\geqslant$ 17 wk resulted in an increased pooled OR of 2.51 and decreased NNT to 4. No dose effect was seen for active disease, but in the maintenance analysis, the OR increased from 1.2 for those taking 1 mg/kg per day to 4.13 at 2.5 mg/kg per day. Fistula healing in the induction studies (defined as complete closure or decreased drainage) was not reported consistently and numbers were small, but a response rate of 55% for treatment compared to 29% for placebo was seen, with an OR of 4.58. One study that was not included because number of fistulae rather than number of patients with fistulae were reported also showed favorable results: 9/29 fistulae (31%) in patients treated with 6-MP compared to 1/17 (6%) in patients taking placebo closed completely[67]. Steroid sparing effects were seen in both the induction and maintenance meta-analyses, with an OR of 3.86 and 5.22 respectively. Patients under treatment for both active and quiescent disease were also more likely to suffer an adverse event leading to withdrawal from studies, with an OR of 3.01 and 4.36 respectively; these events were typically nausea, allergic reactions including fever and rash, pancreatitis and leukopenia. From these studies, it can be concluded that AZA and 6-MP are effective in both the induction and maintenance of remission for CD, although given that maximal clinical benefit may not be evident for three to four months, use of this medication in active disease is best initially coupled with another induction regimen such as steroids, and further, dosing should be optimized for long-term care.

Candy & Wright conducted what is probably the most cited study included in these meta-analyses and elucidates both of these points. Sixty three patients with

active CD were administered a three month taper of prednisolone while randomized to receive either AZA (2.5 mg/kg) or placebo. Although there was no difference in the number of patients achieving remission at wk 12, 42% of the AZA group compared to 7% of the placebo group were in remission at 15 mo ($P = 0.001$)[68]. Further, several studies have evaluated the maintenance benefits of AZA in "withdrawal" trials. In a 12 mo open trial in which 29 patients in remission on AZA for more than two years (median 37 mo) were randomized to continued AZA or withdrawal of AZA, 11/13 (85%) of patients who continued treatment remained in clinical remission compared to 7/15 (47%) of patients who had not continued AZA ($P = 0.043$). This difference was amplified when a subgroup analysis of patients treated with AZA > 1.6 mg/kg per day was performed: 89% of those continued on AZA remained in remission compared to 33% of those withdrawn from AZA ($P = 0.017$)[69]. A larger, longer randomized, controlled trial enrolled patients who had been maintained in remission on AZA for ⩾ 42 mo. Forty patients were randomized to continue the same dose of AZA and 43 to receive placebo for 18 mo. At the end of the study, three patients in the AZA group compared to nine in the placebo group had relapsed: the hypothesis that placebo was inferior to AZA was not rejected ($P = 0.195$). The authors concluded that for patients maintained in remission on AZA, medication should be continued beyond 3.5 years[70].

There is also expanding evidence that AZA is effective as a post-operative maintenance therapy. In an open-label study, 142 patients who had undergone limited bowel resection and/or stricturoplasty were randomized to receive either mesalamine 3 g/d or AZA 2 mg/kg per day within 2 wk of surgery for 24 mo. While risk of clinical (28% *vs* 17% respectively, $P = 0.2$) or surgical (10% *vs* 6% respectively, $P = 0.5$) relapse was equivalent between the two groups, AZA was more effective in preventing clinical relapse among those patients who had undergone more than one surgery for CD (36% *vs* 13%, $P = 0.03$). In this study, adverse events occurred more frequently in AZA-treated patients and caused more frequent study withdrawal (22% *vs* 8%, $P = 0.04$)[71]. In a double-blind, double-dummy multi-center trial, 131 patients who had undergone ileocolonic resection were randomized to daily 6-MP 50 mg, mesalamine 3 g or placebo and were assessed clinically, endoscopically and radiologically at regular intervals over 24 mo. 6-MP was superior at preventing clinical relapse (77%) *vs* mesalamine (58%) or placebo (50%) ($P = 0.045$ for 6-MP *vs* placebo) and endoscopic recurrence (63%, 63%, 43% respectively, $P = 0.03$ 6-MP *vs* placebo) over two years[22].

Thus, treatment with AZA or 6-MP is usually of an "indefinite" duration for patients who have responded. A recent, large European retrospective review of patients treated long-term with AZA demonstrated that in patients with CD, risk of relapse was not greater in patients who discontinued therapy after three to four years, although treatment beyond this time frame improved clinical activity and decreased steroid requirements. The authors conclude that for asymptomatic, steroid-free patients, it may be reasonable to consider discontinuing medication after three to four years of treatment[72].

While thus far treatment with 6-MP or AZA has often been reserved for patients who have required steroids on more than one occasion, there may be benefit to starting these medications earlier in the disease course. A pediatric study randomized children with CD diagnosed within the previous 8 wk to receive 6-MP or placebo for 18 mo, each given with concomitant prednisone. Similar to the Candy study the short-term remission rates were not different between the groups, although patients in the placebo group relapsed significantly more than the 6-MP group (47% *vs* 9%, $P = 0.007$) and required more steroids and for a longer duration[73].

Increased risk of lymphoma with 6-MP and AZA has been debated, with discrepant findings among large series. A recent meta-analysis of six studies ($n = 3891$) showed a four-fold increased risk of lymphoma in IBD patients treated with 6-MP or AZA as compared to the general population: this translated to needing to treat over 4300 patients aged 20-29 and 355 patients aged 70-79 to cause one additional case of lymphoma per year. It is unknown whether this risk relates directly to the medication or to the severity of the disease[74]. Increased risk of hematologic malignancies has also been associated with prolonged leucopenia in IBD patients on 6-MP[75], and EBV-positive lymphomas have also been found more frequently in patients exposed to 6-MP or AZA[76]. The risk of infection with these medication ranges between 0.3%-7.4%[77] and include herpes viruses, human papilloma virus and upper respiratory infections. Physicians prescribing 6-MP and AZA should understand how thiopurine methyltransferase (TPMT) activity affects metabolism of these drugs and should monitor for potential leukopenia and/or hepatotoxicity on a quarterly basis. Measurement of the active metabolite 6-TG may be useful in guiding dosage of these medications.

Methotrexate (MTX)

Methotrexate is a folate analog and reversible competitive inhibitor of dihydrofolate reductase (DHFR). Methotrexate interferes with DNA synthesis and also has multiple anti-inflammatory effects including decreased pro-inflammatory cytokine production and lymphocyte apoptosis[78]. Two exploratory, open-label trials in medically-refractory CD patients with oral[79] or intramuscular (IM)[80] MTX led to the large, multicenter study by Feagan *et al*, in which 141 steroid-refractory patients with active CD were randomized to MTX 25 mg or placebo, intramuscularly over 16 wk. Prednisone was stabilized at 20 mg/d and subsequently tapered over 10 wk. After four months, 39.4% in the MTX group compared to 19.1% in the placebo group had achieved remission (CDAI ⩽ 150 and discontinuation of steroids)[81]. Patients taking MTX suffered significantly more adverse events than the placebo group (16/94) leading to study withdrawal in 17% compared to 2%, although the majority of these side effects were either nausea or asymptomatic liver test abnormalities[81]. Two smaller randomized controlled trials in patients with chronic active disease that compared oral

MTX (12.5 and 15-22.5 mg/wk) did not demonstrate differences in remission rates[82] or flares[83]. More than likely, these unfavorable results are attributable to low, oral dosing with smaller sample sizes as compared to the larger trial. Indeed the bioavailability of oral MTX has been shown to have great variability, averaging 73% that of subcutaneously administered medication[84]. Retrospective data have also reported comparable remission rates to those of Feagan[85-87]. When compared to AZA (2 mg/kg per day) or 6-MP (1.5 mg/kg per day), MTX (25 mg IM changed to po after 3 mo or 15 po/wk) yielded equal rates of remission[88,89], and oral MTX (15 mg/wk) resulted in higher remission rates than 5-ASA 3 g/d (80% *vs* 14%, $P < 0.01$)[89].

MTX also maintains remission in CD. Seventy-six patients who achieved remission with MTX 25 mg IM were randomized to MTX 15 mg IM/wk or placebo. At wk 40, 65% of the MTX group were still in remission as compared to 39% of those in the placebo group and fewer patients required prednisone (28% *vs* 58%, $P = 0.01$). There were no serious adverse events and only one withdrawal from the study secondary to nausea[90]. Several retrospective studies have shown comparable rates patients maintained in remission with MTX[85-87,91].

Mycophenolate mofetil

Mycophenolate mofetil is an ester prodrug of mycophenolic acid which not only inhibits synthesis of guanosine nucleotides and thereby indirectly interferes with T- and B-cell activity, but also inhibits growth of intestinal smooth muscle and synthesis of fibronectin and thus, theoretically could decrease stricture formation. A randomized controlled trial comparing mycophenolate mofetil to AZA in 70 steroid-dependent CD patients with moderately active disease showed equivalent response rates but those with highly active disease seemed to benefit more from mycophenolate mofetil than AZA[92]. Smaller non-randomized studies or series have yielded a combined response rate of 52% overall and 69% in patients with perianal disease[93].

Tacrolimus

Tacrolimus is a macrolide antibiotic used primarily to prevent allograft rejection in the transplant setting. Similar to cyclosporine, it binds to calcineurin and suppresses transcription of activated T-cells leading to decreased pro-inflammatory cytokines such as IL-2, TNFα and INFγ as well as inducing T-cell apoptosis, modifying expression of IL-10 and TGFβ, and may have local effects on the intestine. In a recent review that pooled data from 22 studies with a combined total of 286 patients who had been treated with tacrolimus, promising results in fistulizing disease, unresponsive CD and UC as well as extra-intestinal manifestations were reported[94].

BIOLOGIC AGENTS

Infliximab

Infliximab (Remicade® Centocor, Malvern PA) is a chimeric (75% mouse/25% human) anti-TNFα monoclonal antibody; TNFα mediates multiple pro-inflammatory processes central to the pathogenesis of IBD. The first study that defined efficacy of infliximab in the treatment of active CD randomized patients with moderate-severe, medically-refractory, disease to receive a single infusion of placebo or 5, 10 or 20 mg/kg of infliximab. Seventeen percent, 81%, 50% and 64% of patients respectively had a response (CDAI decrease ⩾ 70 points) at wk 4 ($P < 0.001$ for all infliximab patients *vs* placebo). Overall, 33% of all infliximab patients compared to 4% of placebo achieved remission at wk 4 ($P = 0.005$). While significantly more infliximab patients maintained a response at 12 wk, 37% had relapsed, suggesting that a single dose was insufficient[95]. Those patients who had an initial response to the single infusion were subsequently randomized to receive continued dosing with 10 mg/kg every 8 wk or placebo. After 44 wk, 53% of the infliximab group were in remission compared to 20% of the placebo group ($P = 0.013$)[96].

The ACCENT I study expanded on the potential maintenance benefits of infliximab after an initial response. In the trial, 573 patients received a 5 mg/kg intravenous (Ⅳ) infusion of infliximab at wk 0, after which they were assessed for clinical response by CDAI (decrease in score ⩾ 70 and a 25% reduction in total score). Three hundred and thirty five patients (58%) met this criterion and were randomized to one of three treatment groups: placebo at wk 2 and 6 and then every 8 wk (group Ⅰ), infliximab 5 mg/kg on the same schedule (group Ⅱ) or 5 mg/kg at wk 2 and 6 followed by 10 mg/kg every 8 wk (group Ⅲ). Treatment was continued for 46 wk. At wk 14 or later, patients in all groups who initially had response and then worsened were allowed to cross over to active episodic retreatment (infliximab 5, 10 or 15 mg respectively for groups Ⅰ, Ⅱ, and Ⅲ given on an "as needed" basis). At wk 30, 21% of patients in group Ⅰ, 39% in group Ⅱ ($P = 0.003$) and 45% in group Ⅲ ($P = 0.0002$) respectively were in remission, while median time to loss of response was reported as 19, 38 ($P = 0.002$) and more than 54 wk ($P = 0.0002$) respectively. Significantly more patients in groups Ⅱ and Ⅲ combined (29%) compared with group Ⅰ (9%) had discontinued steroids at wk 54, and fewer hospitalizations and surgeries related to CD occurred in the maintenance therapy groups. There were no differences in serious adverse events between the three groups[97]. A recently published endoscopic sub-analysis of the ACCENT Ⅰ trial showed that scheduled maintenance therapy compared to episodic treatment resulted in greater improvement in mucosal ulceration and higher rates of mucosal healing although the correlation between clinical and endoscopic responses was weak[98].

Infliximab is also effective in the treatment of fistulizing CD. In an initial induction trial, 94 patients with actively draining perianal or abdominal fistulas were randomized to receive three infusions at 0, 2, and 6 wk of placebo, 5 or 10 mg/kg infliximab. Twenty six percent, 68% and 56% of patients respectively achieved reduction in drainage from greater than 50% of fistulas ($P = 0.002$ and $P = 0.02$). Only 13% on placebo compared to 55% and 38% of patients on infliximab had closure of all fistulas ($P = 0.001$ and $P = 0.04$)[99]. In the ACCENT Ⅱ study, 306 patients with one or more draining abdominal or perianal fistulas (⩾ three months duration) received an induction regimen of three infliximab infusions (5 mg/kg).

One hundred ninety-five patients with a response at wk 10 and 14 as well as 87 with no response were randomized to placebo or infliximab (5 mg/kg) every 8 wk to wk 54. Time to loss of response was significantly longer for patients in the infliximab group than placebo (> 40 *vs* 14 wk, $P < 0.001$). Furthermore, at wk 54, 36% in the infliximab group compared to 19% in the placebo group had no draining fistulas ($P = 0.009$)[100]. Relapse of perianal disease after cessation of infliximab may occur earlier than in patients with luminal disease[101].

Antibodies to infliximab are known as both ATIs or HACAs (human anti-chimeric antibodies), and have been associated with lower serum drug concentration levels[97,102] and in turn, with decreased efficacy with episodic treatment[102,103]. In the ACCENT I population, however, equal numbers of antibody-positive and negative patients maintained clinical responses[97]. Additionally, although ATIs are also associated with an increased risk of transfusion reactions[102,103], most ATI positive patients will not have a reaction after re-treatment with infliximab and therefore ATI should not be routinely tested in the absence of loss of response or an infusion reaction[104]. Risk of antibody formation may be decreased by the three-dose induction followed by maintenance therapy[97,103], concomitant use of steroids and/or immunomodulators[97,99,102,103], and pretreatment with hydrocortisone[103]. Sex, location of disease, and smoking status does not appear to correlate with development of ATI[102].

Approximately 30% of patients have no response to infliximab and not all responders have a complete response. As reviewed by Rutgeerts and colleagues, positive predictors of response include elevated CRP, non-stricturing and pure colonic disease subtypes, and concomitant use of immunomodulators[105]. AZA or 6-MP are the immunomodulators most commonly paired with infliximab for CD and it is not clear if the higher response rates seen in combination therapy compared to infliximab alone represents an effect of decreased antibody formation alone or combined efficacy *via* other mechanisms. In contrast to IBD, infliximab has been used concomitantly with MTX in rheumatoid arthritis and a small pilot CD study showed that MTX dosed concomitantly with infliximab may increase remission rates, speed time to remission and decrease steroid use as compared to infliximab monotherapy[106]. Smoking has been found to be a negative predictor of response in two studies[107,108], but surprisingly not in one of the larger studies to examine factors influencing response to infliximab[109].

There has been considerable debate as to whether duration of infliximab treatment must necessarily be life-long or "indefinite," or whether episodic treatment may be a viable alternative. While the clinical and endoscopic benefits of maintenance therapy are demonstrated by ACCENT I and Ⅱ, it has been proposed that the traditional three-dose infliximab induction regimen 0, 2, and 6 wk could serve as a bridge to AZA, but this strategy appeared effective for only six to twelve months[110]. Thus, currently infliximab continues to be recommended for an indefinite period. Another emerging debate is how to position infliximab in the Crohn's treatment algorithm since current regulatory approvals have reserved indications for infliximab for patients who are steroid-refractory or dependent despite immunomodulator therapy. Some argue that this pyramid should be turned upside-down to position infliximab closer to the top, as it has been demonstrated that in steroid-free patients, initial treatment with infliximab and AZA compared with steroids and later addition of AZA leads to significantly more patients in remission and off steroids at 26 wk (60% *vs* 41%, $P = 0.03$) and mucosal healing[111]. If treating early in the disease course with infliximab proved to be disease-modulating, then the "top-down" approach could prove to be the better option to treat those patients on the brink of needing steroids. Arguments against this strategy include the economic costs and the possible safety risks[112].

The safety of infliximab also remains a significant concern with potential serious adverse events including infusion reactions, opportunistic infections including tuberculosis, non-Hodgkin's lymphoma (NHL) and other malignancies, as well as death. A true risk has been difficult to calculate, since most clinical trials did not have continuous placebo arms but instead, were cross-over designs or employed episodic treatment regimens such that most patients were exposed to infliximab at some point[113]. Serious infections were reported in 4% of patients overall in ACCENT I [97] and infliximab-related infections were seen in 8% of a large Mayo ($n = 500$) cohort study of infliximab-treated patients, half of which were serious[114]. As of February 2005, 709 cases of reactivated TB had been reported with infliximab, including 62 deaths[105]. The risk of lymphoma and other malignancies has been difficult to elucidate. The ACCENT I and the Mayo cohort study reported extra-colonic malignancy rates of 1% and 1.5% respectively, but a causal link to infliximab is unclear. CD patients overall likely have a slightly higher risk of NHL[115,116] and squamous cell cancer[116,117].

The TREAT registry has enrolled over 6000 patients from community and academic practices who have been classified in two groups: those who had received infliximab and those who had been treated only with other therapies. The infliximab and non-infliximab patients had similar risks of death, lymphoma and other malignancies; risk of serious infection was slightly higher in the infliximab-treated patients but Cox proportional hazard analysis later found that this risk was independently associated with steroid and narcotic use[118]. In contrast, a recently published decision analytic model projected a slightly increased rate of lymphoma and death in those treated with infliximab compared to those treated with standard therapy, although more quality-adjusted life years were demonstrated in the infliximab group[119]. Twelve cases of hepatosplenic T-cell lymphoma, a rare and incurable type of lymphoma, have been reported in a largely pediatric population (ages 12-31) on combination infliximab and 6-MP or AZA therapy; this association has led to a heightened concern for using these medications concomitantly especially in children, and studies are ongoing to better understand efficacy and safety issues with regard to combination *vs* single agent therapy.

Adalimumab

Adalimumab (D2E7, Humira®; Abbott Laboratories,

Chicago, IL) is a subcutaneously administered recombinant human IgG_1 monoclonal antibody that binds with high specificity and affinity to human TNFα and consists of human-derived heavy and light chain variable regions and human IgG_1 constant region. Adalimumab is now approved in the US and Europe for the treatment of CD. Two open-label trials treated patients with adalimumab who had previous exposure to infliximab. In the first, 24 patients who had lost responsiveness or developed intolerance to infliximab were treated with an initial dose of adalimumab 80 mg and then 40 mg every other week for 12 wk. Although 79% required dose escalation to 40 mg weekly, clinical remission and response at wk 12 was seen in 29% and 59% respectively[120]. In the second trial, 15 patients with attenuated response to infliximab were treated for six months with the same schedule of adalimumab as in the first study. Of the 13 patients who completed the trial, 54% had a complete response, 31% had a partial response, and 73% were able to discontinue steroids[121]. Most recently, the CLASSIC- I trial randomized 299 moderate to severe CD patients naïve to anti-TNF therapy to one of three dose combinations administered at wk 0 and 2 (160/80 mg, 80/40 mg, or 40/20 mg) or placebo. At wk 4, 36% (P = 0.001), 24% (P = 0.06), and 18% (P = 0.36) in the adalimumab groups, respectively, were in clinical remission compared to 12% in the placebo group[122]. Fifty-five patients who were in remission at wk 4 of CLASSIC I were randomized to receive continued adalimumab 40 mg every other week, weekly or placebo for up to one year as part of the CLASSIC Ⅱ trial in which 74%, 83% and 44% of patients, respectively, maintained remission at wk 56[123]. Similar to the ACCENT I study with infliximab, immunomodulator therapy again did not alter these results[124]. Finally, the CHARM trial (n = 854) examined adalimumab induction and maintenance efficacy in patients with moderately to severely active CD. An 80 mg dose at week zero and 40 mg dose at wk 2 were administered to all patients, with 499 (58%) achieving clinical response and then randomized to placebo, adalimumab 40 mg every other week, or 40 mg weekly through wk 56. Significantly higher rates of remission were seen in the adalimumab groups compared to placebo at both wk 26 (40% and 47% *vs* 17%, $P < 0.001$) and wk 56 (36% and 41% *vs* 12%, $P < 0.001$). The adalimumab groups also had significantly more steroid discontinuation and complete fistula closure. Safety data was comparable to other TNF therapy[125].

Certolizumab

Certolizumab pegol or CDP870 (UCB; Smyrna, GA) is a monoclonal humanized anti-TNFα antibody Fab' fragment linked chemically to polyethylene glycol (PEG). In contrast to infliximab and adalimumab the antibody fragment does not induce apoptosis[126]. Certolizumab has been evaluated in both induction and maintenance trials for CD[126,127]. In 92 patients with moderate to severe CD randomized to a single intravenous dose of 1.25, 5, 10 or 20 mg/kg of CDP870 or placebo, the primary endpoints of clinical response or remission after four weeks were not different between treatment groups and placebo, but the remission rate at wk 2 was 47% in the 10 mg/kg group compared to 16% in the placebo group (P = 0.041)[127]. The PRECISE 1 study compared subcutaneous certolizumab (100, 200 or 400 mg) to placebo administered at wk 0, 4, and 8 in 292 patients with moderate-severe CD. While all doses of certolizumab produced significant clinical benefit over placebo at wk 2, 400 mg had the strongest effect at all time points, most markedly at wk 10 (52.8% *vs* 30.1%, P = 0.006); however, no statistical significance in clinical response was seen at wk 12, the primary endpoint. When re-analyzed according to stratification by C-reactive protein level (> 10 mg/L), the 400 mg group had a significantly better response at wk 12 (53.1% *vs* 17.9%, P = 0.005) that was attributed to a lower placebo response rate than those patients with a CRP < 10[126]. In the PRECISE 2 trial, patients who responded to a 400 mg induction dose at wk 0 and 2 (428/668, 64%) were randomized to receive 400 mg certolizumab or placebo every 4 wk for 26 wk. Significantly more patients in the certolizumab arm achieved clinical response (62.8% *vs* 36.2%, $P < 0.001$) and remission (47.9% *vs* 28.6%, $P < 0.001$) at wk 26[128]. Safety and tolerability were similar to other anti-TNF agents, although patients treated with certolizumab had lower rates of autoantibody formation.

Fontalizumab

Interferon γ is cytokine with wide-ranging proinflammatory activity implicated in both animal models of colitis and found to have mucosal elevations in CD. Fontalizumab (Protein Design Labs Inc, Fremont, CA, USA) is a humanized form of mouse antihuman interferon γ antibody recently studied in CD. A controlled trial randomized 133 patients with moderate-severe CD to receive one or two doses of fontalizumab 4 mg/kg, 10 mg/kg, or placebo (28 d apart). Although no differences in response were demonstrated with single dose therapy, in those receiving two doses, response rate at d 56 was found to be 69% and 67% for the fontalizumab groups compared to 32% in the placebo groups (P = 0.02 and 0.03 respectively). This difference was more robust in patients with elevated CRP. Adverse rates were similar across treatment and placebo groups, and all serious adverse events except one were related to CD exacerbations[129].

SELECTIVE ADHESION MOLECULE INHIBITORS

Leukocyte emigration from the vascular space to inflamed tissue is a complicated process involving multiple leukocyte-endothelial interactions including tethering, rolling, firm adhesion, spreading, and migration. Leukocyte adhesion to activated endothelium is mediated primarily by the α_4 and β_2 integrins. The α_4 integrin is expressed on all types of white blood cells and can pair with either the β_1 or β_7 subunit. Endothelial ligands recognized by α_4 integrin include vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MadCAM-1); the former is induced at sites of inflammation, whereas the latter is expressed constitutively on the endothelium within Peyer's patches and other gut-associated lymphoid tissues[130].

Natalizumab

Natalizumab is a recombinant humanized antibody derived from a murine monoclonal antibody (AN100226m) (95% human and 5% mouse-derived) and targets human α_4 integrin. Antibodies to α_4 integrin have shown efficacy in animal models of multiple sclerosis and colitis[131,132]. The preliminary data supporting the use of natalizumab as an induction agent was equivocal as two trials of natalizumab in CD showed a trend toward clinical benefit with either one or two doses of 3 or 6 mg/kg compared to placebo, but primary endpoints did not reach significance[133,134]. Similarly, ENACT-1 did not demonstrate significant differences in clinical response or remission at 10 wk between CD patients (n = 905) treated with an intravenous infusion (300 mg) of natalizumab and placebo at wk 0, 4, and 8[135]. In contrast, the ENCORE study enrolled only patients with an elevated CRP (n = 509) and showed significantly higher rates of clinical response and remission at all time points in those treated with three doses of 300 mg natalizumab (0, 4 and 8 wk) compared to placebo[136].

More consistent outcomes have been shown in maintenance trials for natalizumab. In ENACT-2, initial responders to natalizumab (n = 339) received natalizumab (300 mg) or placebo every 4 wk through wk 56. Significantly more patients in the treatment group compared to placebo had a sustained response (61% *vs* 28%, $P < 0.001$) and remission (44% *vs* 26%, $P = 0.003$) through wk 36[135] and concomitant use of immunomodulators did not affect efficacy[137]. An open-label extension study of ENCORE showed that 84% of patients who were in remission after one year remained in remission for two years after continued monthly treatment with natalizumab[138].

In early 2005, three cases of progressive multifocal leukoencephalopathy (PML) were reported in patients treated with natalizumab, two of them fatal. Two patients had multiple sclerosis and one had CD. Natalizumab trials were subsequently suspended by the U.S. Food and Drug Administration (FDA) and the drug was removed from the market. A safety trial that included 90% of all CD, multiple sclerosis and rheumatoid arthritis participants from all previous natalizumab clinical trials failed to find any additional cases of PML, and the overall risk of PML was estimated at 0.1%[139]. In June 2006, the FDA approved resumption of natalizumab marketing targeting a restricted distribution program for selected MS patients.

MLN02

MLN02 is a humanized monoclonal antibody which specifically recognizes the α_4B_7 heterodimer but does not cross-react with the individual component monomers[140]. The major ligand for α_4B_7 is MadCAM1, and therefore this antibody should be gut-specific in theory. Although clinical and endoscopic efficacy has been demonstrated in patients with UC, patients with mild to moderate CD (n = 185) who received two doses of either 0.5 or 2.0 mg/kg at 0 and 29 d did not achieve the primary endpoint of clinical response at two months. On the other hand, clinical remission was seen in 36.9% of the 2.0 mg/kg group compared with 20.7% of the placebo group ($P < 0.05$)[141].

OTHER BIOLOGIC AGENTS

Visilizumab

Visilizumab (Nuvion™, Protein Design Labs) is a humanized IgG_2 monoclonal antibody (HuM291) to the CD3ε chain of the T-cell receptor expressed on activated T-cells. Designed to capitalize on the potent immunosuppressive effect of OKT3 (a mouse monoclonal antibody used primarily in the transplant setting), it minimizes the anti-mouse antibody response and also the adverse effects of the cytokine release syndrome. While trials in UC have demonstrated safety and efficacy, only one small open-label trial in medically-refractory (including infliximab) CD patients (n = 14) given two doses of visilizumab10 μg/kg on d 0 and 1, 58% and 33% experienced clinical response and remission respectively on d 89, with the mean prednisone dose dropping from 19 mg/d at baseline to 4 mg/d[142].

Anti IL-6 receptor antibody

IL-6 is another cytokine that plays a central role in the inflammatory process of CD. A monoclonal antibody to IL-6 receptor (IL-6R) has been shown to decrease expression of adhesion molecules and multiple pro-inflammatory cytokines in animal models of colitis. A randomized pilot study of humanized anti IL-6R (MRA) in 36 patients with active CD found that those given a biweekly infusion of MRA had an 80% clinical response rate compared to 31% of placebo patients ($P = 0.019$), although endoscopic and histologic examination showed no differences[143].

Anti IL-12 antibody

Interleukin-12 is an important cytokine in the Th1-mediated inflammatory response. A monoclonal antibody targeting IL-12 has been evaluated in a randomized trial in which uninterrupted weekly dosing at 3 mg/kg for seven weeks yielded higher response rates than placebo (75% *vs* 25%, $P = 0.03$), but a statistically significant difference was lost at 18 wk (69% *vs* 25%, $P = 0.08$). The more robust clinical response in the anti-IL-12 group was paralleled by decreases in colonic mononuclear cell secretion of IL-12, INF-γ, and TNFα[144].

Thalidomide

Because of its anti-TNFα and anti-IL-12 properties, thalidomide has been studied in two small open-label trials in mixed IBD populations, with the majority of patients in each achieving either clinical response or remission[145,146]; use of this medication is severely restricted because of its well-known teratogenicity and it is further limited by side effects of sedation and mood disturbances.

IMMUNE STIMULATION

Although immune dysregulation is believed to be a part of the pathogenesis of IBD, an alternative hypothesis proposes that an altered innate immune response is inherent to the etiology of CD. Based upon positive results in other disorders of neutrophil function, granulocyte-

macrophage colony-stimulating factor (GM-CSF), a myeloid growth factor that stimulates the growth and function of phagocytic cells, has been studied in CD. One hundred and twenty-four patients were randomized to receive sargramostim (GM-CSF) 6 mcg/kg per day subcutaneously or placebo for 8 wk: while the primary endpoint of a clinical response (defined by a decrease in CDAI of $\geqslant$ 70 points) was not met, significantly more patients in the sargramostim group reached the secondary endpoints of a decrease in CDAI of $\geqslant$ 100 points (48% *vs* 26%, $P = 0.01$) and remission at d 57 (40% *vs* 19%, $P = 0.01$). The sargramostim group suffered from significantly more injection site reactions and experienced more bone pain[147].

PROBIOTICS AND HELMINTHS

The theory of dysbiosis maintains that a decrease in protective or "good" bacteria and a concomitant increase in harmful or "bad" bacteria contribute to the pathogenesis of IBD. As a result, probiotics have been studied as both induction and maintenance treatment in CD. Open label and small randomized controlled trials using various preparations of probiotics have shown inconsistent results as summarized by Rioux and Fedorak[148]. Large randomized placebo controlled trials are needed in order to determine true efficacy. Similarly, observations that IBD is uncommon in developing countries where helminthic colonization is prevalent and that helminths downregulate Th1 immune responsiveness have led to trials utilizing non-pathogenic helminthes in an attempt to treat UC and CD. An open-label trial of *Trichuris suis* (porcine whipworm) has been studied as a therapy for CD in which 29 patients with active CD ingested 2500 live *Trichuris suis* ova every 3 wk for 24 wk: as defined by CDAI scores, 79% responded and 72% remitted[149]. While results from this study are intriguing, a controlled trial again is essential before deeming worm therapy beneficial.

ULCERATIVE COLITIS

Aminosalicylates

Aminosalicylates (5-ASA) remain the first-line therapy for both induction and maintenance of mild-moderate UC. The efficacy of sulfasalazine specifically has been well-established, but dose-dependent intolerance to the sulfa moiety limits its use in up to one-third of patients[150]. Therefore, non-sulfa-containing 5-ASA agents have been studied as well. Recent Cochrane reviews analyzed the effectiveness of these newer 5-ASA medications both in comparison to placebo and sulfasalazine for the induction and maintenance of remission in UC. Twenty one randomized controlled trials ($n = 2124$) of 5-ASA were included in the induction meta-analysis, nine comparing 5-ASA to placebo and 12 to sulfasalazine[151]; results were reported in terms of failure rates. 5-ASA provided benefit over placebo in the induction of remission, with a pooled OR of 0.53 overall and 0.36 when only the Asacol trials were included. While 5-ASA was better than placebo across all dosage ranges, there was a trend toward a dose-effect. 5-ASA was also more likely to elicit a global or clinical response than placebo, with a pooled OR of 0.40 and higher doses yielding better results, $P = 0.002$. 5-ASA was also superior to placebo at inducing endoscopic remission, but only at doses $\geqslant$ 3 g/d. No significant differences were found between 5-ASA and sulfasalazine in induction of remission or response, although a trend towards 5-ASA superiority was observed. Significantly more patients taking sulfasalazine withdrew from studies secondary to adverse events, with an OR of 0.34; it should be noted that tolerance to sulfasalazine was an inclusion criteria for most of the studies, which may have made this effect less robust.

Sixteen trials ($n = 2479$) were included in the 5-ASA maintenance of remission meta-analysis, five comparing 5-ASA to placebo and 11 to sulfasalazine[152]. 5-ASA was more effective than placebo in maintaining endoscopic or clinical remission, with an OR of 0.47; a dose effect was not observed. Sulfasalazine was superior to 5-ASA in the maintenance of remission in trials of six month duration, with an OR of 1.29, but the statistical significance was lost when only studies with endpoints at 12 mo were included. In subgroup analyses by specific 5-ASA preparation, only olsalazine was found inferior to sulfasalazine, likely secondary to the greater number of adverse events (the most common being diarrhea) and subsequent withdrawals in patients receiving this medication. The authors stated that conclusions could not be reached with regard to other 5-ASA preparations. Save for olsalazine, there were no differences in adverse events between 5-ASA when compared to placebo or sulfasalazine. If sulfasalazine truly has superiority over 5-ASA in maintenance of remission (beyond olsalazine), the authors conjecture that unknown pharmacologic effects of the sulfapyridine moiety previously thought to function only as a carrier of 5-ASA to the colon, could contribute to this finding.

5-ASA formulations

There is no definitive data to suggest that one 5-ASA preparation is superior to another. In one study, balsalazide 6.75 g/d (Colazal®, Salix Pharmaceuticals, Morrisville, NC, USA) was found to induce remission in a greater number of patients with active moderate-severe UC than equivalent doses of Asacol 2.4 g/d (62% *vs* 37% at 12 wk, $P = 0.02$). Further, the median time to complete symptom relief was significantly shorter in the balsalazide than mesalamine group (10 *vs* 25 d, $P = 0.004$)[153]. Two subsequent studies comparing these same medications at the same doses did not demonstrate differences in primary endpoints of rectal bleeding and at least one other sign or symptom at wk 8[154] or symptomatic remission at wk 8[155]. Secondary endpoints showing balsalazide to have a faster time to onset[154,155] or better effect in new onset left-sided disease[155] cannot be considered more than preliminary given that primary endpoints were not met[156]. Further, no differences between balsalazide and sulfasalazine or Salofalk® (a delayed-release pH dependent mesalamine formulation) have been found[156]. Additionally, as will become evident below, a suboptimal dose of Asacol® was used in these studies[157]. As well, non-traditional clinical assessments were employed[158], and the Asacol® was not equivalent to that used in the US and in pivotal trials, as demonstrated by *in vitro* dissolution experiments[159].

Pharmacokinetic data in healthy patients demonstrates no differences in systemic absorption of 5-ASA between Asacol and balsalazide at equimolar doses[160]. Therefore, choice of 5-ASA agent should be based upon tolerability, ability to titrate dose to effect and cost.

Dose-effect

The recent ASCEND trial showed a dose-effect based on severity of disease. An overall response rate of 72% at wk 6 was found in patients with moderate activity (n = 268) treated with mesalamine 4.8 g/d (investigational 800 mg tablet, Procter and Gamble Pharmaceuticals, Mason, OH) compared to 59% in those receiving Asacol® 2.4 g/d (P = 0.036). No difference in response rate in patients with mild disease was demonstrated with the two different doses[161]. Patient compliance in taking 5-ASA may be enhanced by a higher dose tablet, SPD476 (1.2 g/tablet) which uses both a gastro-resistant polymer film to delay release of active drug until it reaches the terminal ileum and Multi Matrix System (MMX) which helps deliver 5-ASA evenly throughout the colon. MMX 2.4 g/d and 4.8 g/d are superior to placebo in the induction of remission in mild-moderate UC[162].

Topical mesalamine

Rectal mesalamine induces remission more effectively than placebo or topical steroids in distal UC[163,164], although both medications taken concomitantly are superior to mesalamine alone[164]. Topical mesalamine is superior to placebo and at least as effective as oral mesalamine in the maintenance of remission for distal UC[163,164].

STEROIDS

For patients without sufficient response to 5-ASA agents or those with moderate-severe disease, glucocorticosteroids have remained the foundation for inducing remission in UC since the early 1950s when Truelove and Witts reported significant benefit for cortisone over placebo[165]. For mild to moderate UC, a dose effect for prednisone 20-60 mg/d has been reported, but doses greater than 60 mg/d confer no additional benefit[166]; further, there does not appear to be a difference between once daily and divided dosing[167]. For those with severe colitis or not responding to oral regimens, parenteral steroids are administered. While mineralocorticoid and anti-inflammatory potencies vary, no data suggests one preparation is superior to another; methylprednisolone 40-60 mg/d or an equivalent dose of hydrocortisone is the most commonly used. Adrenocorticotropic hormone (ACTH) promotes endogenous corticosteroid production and may have benefit in steroid-naïve patients, but is no longer commonly utilized due to the potential for adrenal hemorrhage[35]. Pulse-dose steroids in the form of dexamethasone 100 mg/d have shown efficacy in a small open-label trial[168], but a controlled trial has not yet been conducted and a recent systematic review suggests the absence of a dose-response above the equivalent of 40 mg of prednisone[169]. Dividing intravenous bolus dosing is equally effective to a continuous infusion[170].

Predictors of decreased response rate to steroids and increased risk for colectomy include greater severity and extent of colitis[171,172], and most recently persistence of stool frequency > 8/d or CRP > 45 mg/L beyond three days of treatment[173]. Higher levels of glucocorticoid receptor beta (GRβ) have also been associated with glucocorticoid resistance in several studies[174-176]. Of those patients who will respond to IV steroids, the majority do so within five days[177], but most practitioners will continue treatment for 7-10 d[35].

NON-SYSTEMIC STEROIDS

With the multitude of adverse effects of systemic steroids, non-systemic steroids have generated great interest in UC given their high-first pass metabolism and minimal toxicity. Because most of budesonide is released in the distal ileum and proximal colon, making it an effective medication for the treatment of CD in this location, its role in UC is likely very limited, although one study showed equal efficacy to prednisolone in those with left-sided or extensive colitis[178]. An oral formulation of beclomethasone dipropionate (BDP) coated with Eudragit L preventing gastric dissolution and releasing at pH 6.0 for delivery in the terminal ileum and throughout the colon was evaluated in a single-blind randomized trial enrolling 177 patients with mild-moderate UC[179]. Patients who received BDP 5 mg/d for 4 wk had equivalent reductions in mean disease activity index or DAI (assessment of clinical and endoscopic response) and clinical remission rates as those receiving 5-ASA 2.4 g/d, although a significantly greater improvement in DAI was seen in those with extensive disease in the BDP group. BDP was also found to have an additive effect when given in conjunction with 5-ASA[180].

Non-systemic steroid enemas are beneficial in the treatment of active distal UC. Budesonide enemas have significantly higher remission rates than placebo, between 19%-51% with daily 2 mg/100 cc dosing[181,182]; higher doses do not appear to be of greater benefit but may result in more adrenal impairment[182]. While 2 mg twice weekly was not any more effective than placebo in the maintenance of remission[182], it is possible that the optimal dosage for preventing relapses has not been defined. When compared to topical mesalazine (1 g/100 mL per day), budesonide enemas were equally effective in improving histologic and endoscopic scores but clinical remission rates were higher in the mesalazine group[183]. Budesonide enemas are equally or more beneficial than traditional steroid enemas in clinical, endoscopic and histologic measures and induce less adrenal suppression[184-186]. Budesonide foam and enemas resulted in similar clinical remission rates in a large double-blind, double-dummy trial[187]. Similarly, BDP enemas have shown equal efficacy to 5-ASA[188] and prednisolone enemas[189], but are not associated with adrenal axis suppression[189]. The combination of 5-ASA and BDP was superior to either alone[188].

IMMUNOMODULATORS

AZA and 6-MP

The first reported use of AZA in the treatment of UC was in the 1960s, but results from initial controlled trials

in the mid-1970s did not show clinical or endoscopic benefits over placebo[190,191]. However, it became apparent that treatment with AZA consistently permitted significant steroid reduction compared to placebo[191,192]. Patients with UC in remission on AZA ⩾ six months relapsed at a higher rate over one year when withdrawn to placebo (59%) as compared to those who continued AZA (36%), $P = 0.039$; this effect was more pronounced with longer pre-trial remission rates[193]. Later retrospective studies also reported the steroid-sparing effect of AZA and 6-MP[194-197], higher relapse rates with cessation of 6-MP[194,195], and fewer colectomies in those patients maintained on AZA[196,197]. Length of treatment appears to correlate with efficacy: a large retrospective review of both CD ($n = 272$) and UC ($n = 346$) patients treated with AZA found a remission rate of 87% in those patients treated more than six months, compared to 59% overall. Other factors predictive of remission were the diagnosis of UC (*vs* CD), lower white blood cell (WBC) or neutrophil count, a higher mean corpuscular volume and older age. On continued AZA, 95%, 69%, 55% of patients at 1, 3 and 5 years respectively were maintained in remission compared to 63%, 44%, and 35% after discontinuation of AZA; risk of relapse was lower in those with WBC ⩽ 5.0×10^5 ($P = 0.03$)[198].

Compared to mesalazine at a dose of 3.2 g/d, significantly more steroid-dependent patients treated with AZA, 2 mg/kg, achieved both clinical and endoscopic remission as well as steroid discontinuation (53 *vs* 21%, $P = 0.006$)[199]. The addition of 5-ASA to AZA does not confer greater benefit than AZA alone in the maintenance of remission[200,201] or steroid-withdrawal[200].

METHOTREXATE (MTX)

While an initial small open-label study of MTX in IBD held promise for both CD and UC[80], randomized controlled trials have shown benefit in CD only[81,90]. Although several additional open-label or retrospective studies of MTX in UC showed favorable effect[91,202,203], two randomized controlled trials showed no differences in the induction or maintenance[89,204] of remission between patients given oral MTX 12.5[204] or 15 mg[89] per week compared to placebo. It should be noted, however, that in the definitive study that established the efficacy of MTX in the induction of remission in CD, patients were given 25 mg IM; ideally, a second randomized controlled trial in UC utilizing a higher dose of MTX would be conducted.

CYCLOSPORINE

Cyclosporine (CSA) is a lipophilic peptide with multiple anti-inflammatory effects including downregulation of IL-2, thus inhibiting proliferation and activation of T-helper cells[205]. After the first promising open-label trial in 1990 in which 73% of 15 severe, steroid-refractory UC patients treated with Ⅳ CSA (4 mg/kg) improved over an average of 5.8 d and avoided colectomy[206], a double-blind controlled trial that randomized 20 patients with severe steroid-refractory UC to Ⅳ CSA (4 mg/kg) or placebo showed an 82% response rate in the CSA group at a mean of seven days compared to zero in the placebo group ($P < 0.001$). All five placebo group patients given CSA during the open-label phase responded to treatment and over two-thirds of all responders avoided colectomy at six months[207]. In non-randomized controlled trials, long-term remission rates have been less impressive ranging between 14%-40%[208-212]; "bridging" to AZA after induction with Ⅳ CSA and a short course of oral CSA improves maintenance rates, with 40%-90% of patients avoiding colectomy after 16-78 mo[213-215]. Similarly, in a retrospective review at the University of Chicago, 62% of all UC patients ($n = 42$) treated with CSA avoided colectomy over a mean follow-up of 23 mo. This rate improved to 72% among initial CSA responders and to 80% in initial responders who were later transitioned to 6-MP or AZA[216]. Skipping oral CSA and transitioning directly to (6-MP or AZA) after Ⅳ therapy does not seem to alter long-term outcome[217].

CSA is associated with significant morbidity, including opportunistic infections, neurologic and renal toxicity, hypertension, and rarely, death[218]. Several variations in therapy may decrease the risk of these adverse events without compromising efficacy: the use of low-dose CSA (2 mg/kg)[218-220], oral microemulsion CSA (Neoral®) with 60% bioavailability[221-223], and Ⅳ CSA without concomitant steroids[224]. Higher percentages of band forms on differential WBC count[225], tachycardia > 90 bpm, fever > 37.5℃, elevated CRP > 45 mg/L, and greater than one severe endoscopic lesion are negative predictors of response[226]. Ideally, administration of CSA should be limited to physicians trained or experienced in the use of potent immunosuppressants or transplantaion[227].

TACROLIMUS

UC patients with active refractory moderate-severe disease ($n = 63$) were randomized to tacrolimus dosed to maintain either a high (10-15 ng/mL) or low (5-10 ng/mL) trough or placebo for two weeks with an open-label extension segment. Sixty-eight percent of patients in the high-trough group achieved partial response as measured by the UCDAI (number of bowel movements, bleeding and physician's global assessment) compared to 10% in the placebo group ($P < 0.001$). While 38% in the low-trough group achieved a partial response, this did not meet statistical significance; however, significant differences were found on multiple components of the UCDAI between the low-trough group and placebo. When placebo patients crossed-over to the open-label extension, 58% achieved response ($P = 0.012$). While no differences in overall adverse events were found, patients in the high-trough group experienced more medication-related adverse events than the placebo group[228]. A recent review of tacrolimus in IBD patients also found overall favorable results in the treatment of refractory UC[94].

MYCOPHENOLATE MOFETIL

Few trials have examined the efficacy of mycophenolate mofetil (MMF) in UC. In a six month open-label uncontrolled study, 24 steroid-dependent chronic active IBD patients received MMF 2 g/d and were tapered to

5 mg of prednisone per day by the second three months. Among the 13 UC patients, six achieved remission by three months, but all relapsed during the second part of the study[229]. The results were equally disappointing in the CD population, with only one patient maintaining remission by the end of the study. A retrospective study of 39 largely steroid-dependent and AZA-refractory or intolerant IBD patients given a median dose of MMF 1.5 g/d reported more favorable results, with 40% of patients in remission off steroids after a mean duration of 19 mo of treatment[230]. An open-label study randomized 24 patients with active UC to receive either MMF (20 mg/kg) or AZA (2 mg/kg), each given with a tapering dose of prednisolone over one year. While the AZA group experienced significantly greater decreases in the clinical colitis activity index (CAI) than the MMF group at three and six months, these differences were no longer significant at nine and twelve months. Further, although at almost all time points, more AZA-treated patients were in remission and using fewer steroids than the MMF group, none of these differences were statistically significant[231].

BIOLOGIC AGENTS

Infliximab

While several small studies of infliximab collectively showed equivocal efficacy in UC, the ACT (Active Ulcerative Colitis Trials) 1 and 2 provided definitive evidence supporting its efficacy in this population[232]. ACT 1 patients were refractory or intolerant to steroids and/or AZA/6-MP, while ACT 2 also included those refractory or intolerant to 5-ASA agents as well. In each trial, 364 patients received either placebo or infliximab 5 or 10 mg/kg at wk 0, 2, and 6 and then every 8 wk through wk 46 and 22 with follow-up data collected to wk 54 and 30 respectively. In ACT 1 and 2, infliximab at either dose was significantly more beneficial than placebo at all time points in achieving clinical response and remission, mucosal healing, and discontinued use of steroids. Overall, approximately two-thirds in the infliximab group achieved clinical response and one-third achieved long-term remission, while 22% discontinued steroids. Rates of adverse events were similar between groups, although one case each of tuberculosis and histoplasmosis (the latter resulting in death) as well as three neurologic complications occurred in the infliximab group. ATIs were found in 6% and conferred a mildly higher risk of infusion reaction. Concomitant immunomodulator therapy was associated with a lower rate of antibody formation, but no conclusions can be reached given the small numbers with ATI overall. Five mg/kg is the recommended starting dose given that there were no significant differences found between the two doses.

While the ACT studies have established infliximab as effective treatment for UC in the outpatient setting, the role of infliximab in the treatment of hospitalized patients is uncertain. Forty-five moderate-severe or fulminant UC patients refractory to Ⅳ steroids at 5 and 3 d respectively were treated with a single dose of infliximab 5 mg/kg or placebo. Overall, infliximab patients avoided colectomy within the first three months more often than placebo patients (67% *vs* 29%, $P = 0.017$); however, in a subgroup analysis of those with fulminant colitis compared to placebo, this difference was no longer significant (69% *vs* 47%, $P = 0.276$)[233]. In an open-label trial of infliximab in 12 hospitalized steroid-refractory UC patients, nine underwent colectomy within three months[234]. Two recent studies showed favorable response profiles to infliximab in patients with acute severe UC[235,236], and it has been hypothesized that this subset of patients may be different than those with established disease.

SELECTIVE ADHESION MOLECULE INHIBITORS

Natalizumab (Tysabri®, Elan and Biogen Inc, USA)

Only one open-label trial of natalizumab has been conducted in UC, in which 10 patients with active disease were given a single dose of 3 mg/kg: while significant clinical and quality of life improvement were seen at one month, only two patients entered remission and by 8 wk, 80% of patients required rescue medication[237]. Given the association of PML with this medication in CD and MS patients, the status of future trials is unknown.

MLN02

Compared to those who received placebo, mild-moderate UC patients ($n = 181$) who received two doses of MLN02 0.5 or 2.0 mg/kg over one month experienced higher rates of clinical remission (33% and 32% *vs* 14%, $P = 0.03$), clinical response (66% and 53% *vs* 33%, $P = 0.002$), endoscopic remission (28% and 12% *vs* 8%, $P = 0.007$) and endoscopic improvement (48% and 35% *vs*16%, $P = 0.001$)[140]. Antibodies to MLN02 were found in 44% of patients; of those with titers ⩾ 1:125, 24% had loss of saturation to $\alpha_4\beta_7$ binding sites with the clinical remission rate in this group close to that of placebo. There were no differences in adverse events. MLN02 appears promising, but more research will need to assess long-term response and optimal dosing.

Alicaforsen

Alicaforsen (ISIS 2302, Isis Pharmaceuticals, Inc. Carlsbad, CA) is a 20-base phosphorothioate oligodeoxynucleotide antisense molecule that down-regulates messenger RNA for intracellular adhesion molecule I (ICAM-1), a transmembrane glycoprotein that is up-regulated by pro-inflammatory mediators. ICAM-1 is involved in leukocyte activation and migration and elevated levels in serum and mucosa have been found in animal models and patients with IBD[238]. Parenteral alicaforsen was not effective in CD[239], but enema formulations appear beneficial in UC and pouchitis in small studies. In one trial ($n = 40$), mild-moderate active distal UC patients who received daily alicaforsen enemas at 0.1, 0.5, 2, or 4 mg/mL for one month experienced an overall dose-dependent improvement in disease activity index (DAI) ($P = 0.003$). At three months, DAI in the 4 mg/mL group dropped by 72% compared with 11.5% in the placebo group ($P = 0.016$), and no one in the alicaforsen group needed additional therapy at six months compared to 50% of placebo patients[240]. No serious adverse events were reported.

ANTI-INTERLEUKIN-2 (IL-2)

IL-2 is a cytokine produced by activated T-cells that binds to the high affinity receptor IL-2R in the presence of the α-chain CD25 and thereby perpetuates T-cell proliferation and activation[241]. Further, high levels of IL-2 have been associated with steroid-resistance[242]. Two anti-IL2 antibodies have been evaluated in UC.

Daclizumab (Zenapax, Roche, Basel, Switzerland), is a recombinant humanized monoclonal antibody (IgG_1) to IL-2R. Although a small, open-label pilot study initially held promise for this antibody[243], a recently published randomized controlled trial (n = 159) found no difference in response or remission rates between two doses of daclizumab and placebo given every other week for 8 wk[244]. Basiliximab, a chimeric monoclonal antibody to the IL-2R (CD25) α-chain, induced remission in the majority of 10 steroid-resistant UC patients given a single dose in an open-label trial[241]. Additionally, *in vitro* testing performed in healthy volunteers and quiescent UC patients as part of this study showed that basiliximab reverses steroid-resistance, and thus anti-IL-2 treatment might have particular potential in steroid-resistant patients.

VISILIZUMAB (NUVION)

Visilizumab, an anti-CD3 monoclonal antibody is undergoing evaluation in severe UC. In an open-label phase I trial, 79% and 54% of steroid-refractory UC patients treated with 10 mcg/kg per day (n = 24) for two consecutive days experienced response and remission respectively at d 30, and 100% of those treated with 15 mcg/kg per day (n = 8) achieved both response and remission[245]. Sixty-three percent of patients receiving the higher dose remained in remission at one year. Almost two-thirds of patients experienced symptoms of cytokine release syndrome 1-3 h post-infusion, including nausea, chills, fever, headache and arthralgias. Decreased T-cell levels persisted for a mean of three weeks post-infusion. Because elevations in EBV titers were reported in patients with graft versus host disease who received visilizumab[246], this UC study excluded EBV+ patients, but a large open-label trial including EBV+ patients is ongoing.

INTERFERONS

Interferon-alpha (IFNα)

IFNα has a range of anti-viral, anti-tumor and anti-inflammatory activity, including induction of IL-1 receptor antagonist and soluble TNF receptor p55, and downregulation of Th-2 cytokines[247]. With the recognition that IFNα does not induce colitis flares in those being treated for chronic hepatitis who have co-morbid UC[248,249] IFNs have been studied as treatment for UC.

In a small (n = 28) open-label trial of IFNα2a given subcutaneously for 6-12 mo in UC patients, 93% achieved and maintained clinical and endoscopic remission for two years[250], while another small study showed only short-lived benefit of IFNα2a over prednisolone enemas in treating distal UC[251]. In the only randomized placebo controlled trial, UC patients refractory to 5-ASA agents, steroids or AZA (n = 60) received either weekly pegylated INFα2b 0.5 or 1 mg/kg or placebo: no difference in clinical or endoscopic response was demonstrated, and a high attrition rate was seen in all groups, mostly secondary to lack of efficacy[247]. Thus, while INFα does not appear to exacerbate UC when treating chronic hepatitis, it is not effective as a primary treatment for UC.

Interferon B

Interferon β (INF-β) also has anti-inflammatory properties including the upregulation of IL-10 and IL-1 receptor antagonist and downregulation of TNF and IL-2. INF-β has had varied results in the treatment of UC. In an open-label pilot study, 88% of 25 steroid-refractory UC patients treated with either Ⅳ human natural IFN-β or subcutaneous recombinant IFN-β for a mean of 52 wk achieved remission lasting over a year[252]. While one small randomized trial showed endoscopic benefit in patients treated with IFNβ1 compared to placebo[253], a larger controlled trial failed to show any advantage recombinant IFNβ1a over placebo in clinical response or remission, endoscopic index, or steroid reduction[254].

GROWTH FACTORS

Growth factors may restore the protective and reparative foundation of the colon, and therefore represent a possible therapeutic option for UC. Growth factors that have been identified as potentially beneficial in treating UC include transforming growth factor β (TGF-β), epidermal growth factor (EGF), keratinocyte growth factor-1 and 2 (KGF-1 or 2, also known as fibroblast growth factor 7 or 10). Repifermin is a truncated, purified KGF-2 expressed in *Escheria coli*, and induces the proliferation of intestinal and colonic mucosa and reduces intestinal ulcers and inflammation in animal models[255]. Intravenously administered repifermin (1-50 μg/kg) for five consecutive days did not yield different rates of clinical response or remission at wk 4 compared to placebo in patients with active UC[255]. Among other reasons, the authors suggested that under-dosing and/or under-powering could have accounted for the negative findings. EGF is a mitogenic peptide produced by salivary and duodenal Brunner's glands: topical application is beneficial in wound healing and systemic EGF is useful in treating neonatal necrotizing enterocolitis[256]. An 83% remission rate was demonstrated in patients with mild to moderate left-sided UC (n = 24) randomized to daily EGF enemas for 2 wk compared to 8% in the placebo group ($P < 0.001$); disease activity, endoscopic and histologic scores remained significantly better in the EGF group through 12 wk[256]. Rebamipide is an amino acid analog of 2-(1H)-quinolinone used to treat gastric ulcers in Japan. It aids mucosal healing by stimulating local prostaglandin synthesis and epithelial cell regeneration *via* upregulation of EGF and its receptor, neutrophil suppression, and decreased production of inflammatory cytokines stimulated by NSAIDs and/or *H pylori*[257]. A small open-label trial in which twice daily Rebamipide enemas were given to patients with UC proctitis for one month demonstrated significant clinical, endoscopic and histopathologic improvement[258]. Larger

controlled trials are needed to evaluate this class of therapy in UC.

CURCUMIN

Derived from tumeric, curcumin appears to inhibit NFκB and possesses anti-inflammatory, anti-microbial and tumor-suppressing characteristics; it has been shown to prevent and treat colitis in animal models. Fewer patients with quiescent UC (n = 82) randomized to 5-ASA plus curcumin compared to 5-ASA plus placebo relapsed over six months (2 *vs* 8, $P = 0.04$)[259].

NICOTINE

While CD is exacerbated and more difficult to treat in active smokers, UC by contrast is a disease of non-smokers. Older patients diagnosed with UC are commonly ex-smokers[260]. One study showed that while the risk of developing UC was not statistically different between those who never smoked and active smokers, ex-smokers were at greater risk to develop UC, suggesting that cessation of smoking increases risk[261]. It is speculated that nicotine alters systemic and/or gut immune function in a protective way; the exact mechanism remains unknown[262]. A Cochrane review found that transdermal nicotine (15-25 mg/d for 4-6 wk) induces remission more readily than placebo although benefit was not greater than mesalamine or corticosteroids. More patients experienced side effects (nausea and light-headedness) with nicotine compared to the other medications[263]. In contrast, transdermal nicotine is no more effective than placebo in the maintenance of remission[264], and nicotine enemas are no more effective than placebo in achieving remission in patients with distal UC[265].

APHERESIS

Selective apheresis of leukocytes, including the targeted removal of monocytes, granulocytes, and lymphocytes is a growing area of research in the treatment of UC. Review of leukocyte apheresis studies shows efficacy in inducing remission across various UC populations in small, open trials[266], but the inherent process of apheresis makes controlled studies difficult to conduct. Two larger trials have demonstrated that leukocyte apheresis (n = 76) and granulocyte/monocyte apheresis (Adacolumn®) (n = 69) are equally or more effective than steroids in the induction of remission[267,268], with fewer adverse events[267] and greater steroid-sparing effects[268]. In the only sham-controlled trial to date, 19 patients with moderate to severe UC treated with five weekly sessions of either leukocyte apheresis (followed by every other week for 4 wk) or sham apheresis demonstrated that the leukocyte apheresis group had significantly greater clinical improvement (80%) than the sham group (33%)[269]. Maintenance of remission after apheresis has been equivocal: in one study of 71 patients with active UC treated with leukocyte apheresis, only 27% of those with an initial response (n = 53) maintained remission for more than six months; rapid response to treatment was the only factor correlated with long-term response in multivariate analysis[270]. In another study, however, 26 of 33 patients maintained remission at one year after 11 weekly sessions of granulocyte/monocyte apheresis[271]. Apheresis may be effective in other settings as well, including a small group of patients with toxic megacolon[272], acute pouchitis[273] and a patient with pyoderma gangrenosum[274].

PROBIOTICS

While there is suggestion that probiotics may benefit patients with active UC, data are limited. Open-label studies with VSL #3[275] and *Saccharomyces boulardii*[276] have shown promise, while a small randomized controlled trial of bifidobacterium fermented milk (100 cc/d for 12 wk) in 20 patients demonstrated significant clinical, endoscopic and histologic improvement over placebo[277]. *Bifidobacterium longum* combined with Synergy, a prebiotic (inulin oligofructose growth substrate) showed a trend toward endoscopic improvement over placebo and significantly decreased inflammatory cytokines such as TNFα and IL-1 in the treated group[278]. No difference in relapse rates were seen among 327 patients with quiescent UC given *Escherichia coli* Nissl 1917 compared to mesalamine over 12 mo[279]. Among 187 patients with inactive UC given either *Lactobacillus* GG, mesalamine or a combination of the two treatments, no differences in relapse rates at six or twelve months were seen across the three groups, although treatment with lactobacillus GG alone or together with mesalamine prolonged relapse-free time[280]. A review by Rioux and Fedorak, showed that VSL #3 has also been beneficial in the maintenance of remission for pouchitis[148].

TRICHURIS SUIS

Although the mechanism is unclear, helminthic colonization has been theorized to be protective against the development of IBD based both on epidemiologic and animal model data. In a randomized controlled trial of 54 patients with active UC, those who received *Trichuris suis* ova every 2 wk for 12 wk had a greater response rate (43%) compared to those who received placebo (16.7%), $P = 0.04$[281]. While intriguing, this study has been questioned regarding whether the statistically significant decrease in activity index of the treated group represents a clinically significant difference[282].

CONCLUSION

The treatment of IBD is a burgeoning field: in particular, the introduction of infliximab, an anti-TNFα medication, almost a decade ago, has been the most significant addition to the spectrum of therapeutic options in IBD, which for many years was primarily limited to 5-ASAs, antibiotics, steroids and immunomodulators. Medications may be used either to induce or maintain remission. Choice of therapy depends largely on the severity of disease, and may also be influenced by such factors as disease location, side effects and adverse events, as well as cost. While there is much debate presently regarding "top-down" compared to the traditional "step-up" treatment a reversal of the

"therapeutic pyramid" awaits more data regarding short- and long-term efficacy, safety, and pharmacoeconomic data. Aminosalicylates remain the standard induction and maintenance therapies for UC but have a more equivocal role in CD. Despite a paucity of evidence, antibiotics are also commonly used in CD, especially with colonic and perianal disease. Budesonide is effective as a first-line agent for ileal and/or right colonic CD although maintenance benefits remain to be proven. Conventional steroids induce remission for both CD and UC but are reserved for patients with moderate-severe disease or for those who have failed more first-line therapy. Immunomodulators such as 6-MP and azathioprine, as well as methotrexate, are effective steroid-sparing and maintenance therapies. Cyclosporine or tacrolimus can be effective for severe or refractory UC. Anti-TNF agents have been effective for patients with moderate-severe UC and CD, independent of concomitant medications. Potential side effects, costs and immunogenicity remain issues relating to current and future biologic agents. Novel therapies continue to be explored as the immunopathophysiologic underpinnings of IBD continue to be elucidated and an ultimate etiopathogenesis remains undetermined.

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TOPIC HIGHLIGHT

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Cancer in inflammatory bowel disease

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Abstract

Patients with long-standing inflammatory bowel disease (IBD) have an increased risk of developing colorectal cancer (CRC). Many of the molecular alterations responsible for sporadic colorectal cancer, namely chromosomal instability, microsatellite instability, and hypermethylation, also play a role in colitis-associated colon carcinogenesis. Colon cancer risk in inflammatory bowel disease increases with longer duration of colitis, greater anatomic extent of colitis, the presence of primary sclerosing cholangitis, family history of CRC and degree of inflammation of the bowel. Chemoprevention includes aminosalicylates, ursodeoxycholic acid, and possibly folic acid and statins. To reduce CRC mortality in IBD, colonoscopic surveillance with random biopsies remains the major way to detect early mucosal dysplasia. When dysplasia is confirmed, proctocolectomy is considered for these patients. Patients with small intestinal Crohn's disease are at increased risk of small bowel adenocarcinoma. Ulcerative colitis patients with total proctocolectomy and ileal pouch anal-anastomosis have a rather low risk of dysplasia in the ileal pouch, but the anal transition zone should be monitored periodically. Other extra intestinal cancers, such as hepatobiliary and hematopoietic cancer, have shown variable incidence rates. New endoscopic and molecular screening approaches may further refine our current surveillance guidelines and our understanding of the natural history of dysplasia.

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Key words: Colon cancer; Inflammatory bowel disease; Dysplasia; Chemoprevention; Colonoscopy; Genomic instability

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INTRODUCTION

The two forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic, relapsing inflammation of the intestines. First described in a report by Crohn and Rosenberg in 1925[1], colorectal cancer (CRC) in patients with IBD has long been recognized. Even years after their disease is controlled with medications, IBD patients still live with the fear of developing cancer. CRC is the most common site of cancer in IBD, although cancer in other organs can occur. Together with the hereditary syndromes of familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer, IBD is among the top three high-risk conditions for CRC. To date, our understanding of CRC pathobiology has come from studies of patients with UC more so than Crohn's colitis. This review will focus mainly on the problem of CRC, and then address other cancers such as small intestinal adenocarcinoma, cholangiocarcinoma, and hematologic malignancies.

COLORECTAL CANCER IN PATIENTS WITH IBD

Prevalence and Incidence of CRC

The exact magnitude of the risk of cancer has been difficult to quantify due to various biases and methodological errors in published studies. Early estimates of CRC complicating UC were based on crude percentages and all were from major medical institutions, predominantly tertiary referral centers. Studies from these centers often included a greater proportion of patients who had more severe disease and cancer had already complicated their colitis. These center-based studies often overestimate the risk. Later population-based studies tended to include more patients with limited disease or those who have undergone colectomy and may thereby underestimate the true risk. Based on a 2001 meta-analysis by Eaden *et al*, including 116 studies from around the world, the prevalence of CRC in patients with UC is approximately 3.7% overall and 5.4% for those with pancolitis[2]. In comparison, CRC in Crohn's disease has been less well studied. Early studies showed no statistically significant increase in cancer risk among the Crohn's disease patients. However, the lack of the risk of cancer in these studies was often due to inclusion of all patients with Crohn's disease and failed to correct for the small subsets of those with extensive, longstanding and unresected colonic disease. Hence, when patients with longstanding,

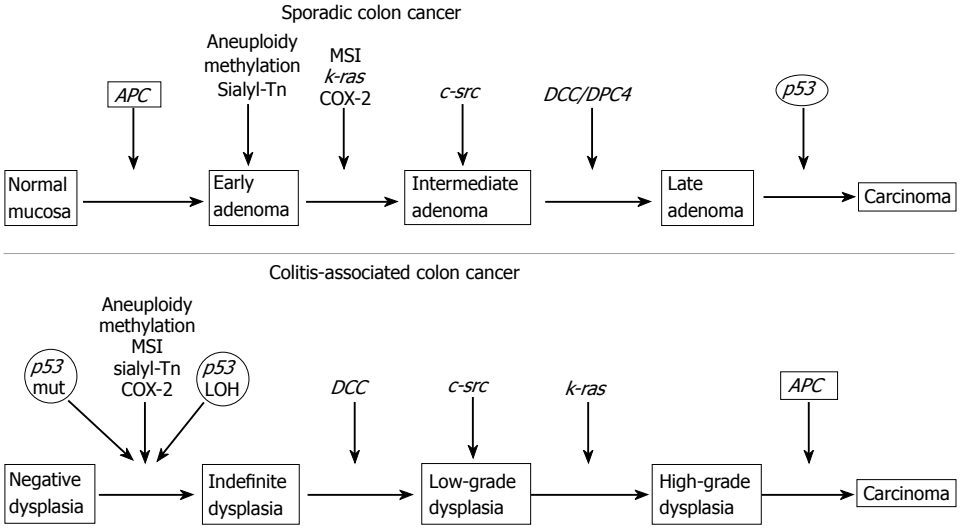


Figure 1 Comparison of molecular alterations in sporadic colon cancer and colitis-associated colon cancer. Mut, mutation. Modified from Ref 71. With permission.

anatomically substantial Crohn's colitis are considered, the risk of CRC is similar between Crohn's colitis and UC[3]. Indeed, a population-based study from Manitoba, Canada found that the risk for colon cancer among patients with both UC and Crohn's colitis is approximately 2-3 fold greater than the general population and that the risk of rectal cancer is increased 2-fold in UC but not Crohn's colitis[4].

Clinical features of colitis-associated CRC

Compared with sporadic colorectal carcinoma (SCC), CRC arising in patients with IBD has several distinguishing clinical features. Colitis-associated colorectal cancer (CAC) affects individuals at a younger age than the general population. CAC progresses to invasive adenocarcinoma from flat and nonpolypoid dysplasia more frequently than SCC. CACs more often have a higher proportion of mucinous and signet ring cell histology. There is background of chronic inflammation in colitis and a higher rate of two or more synchronous primary CRCs. The multifocality of CAC relates to the broader field effect of mucosal inflammation that gives rise to the neoplasia. In some studies, patients were found to have cancer more proximal in the colon. Finally, the sequence of molecular events leading from dysplasia to invasive adenocarcinoma is different from that of SCC (discussed below).

Molecular features of sporadic colon cancer

To place the molecular pathogenesis of colitis-associated neoplasia in proper perspective, it is important to appreciate the molecular events involved in the development of SCC. SCC arises as a result of genomic instability. The two main types of genomic instability that contribute to colon carcinogenesis are chromosomal instability (CIN) and microsatellite instability (MSI), accounting for 85% and 15% of SCC, respectively. Chromosomal instability results in abnormal segregation of chromosomes and abnormal DNA content (aneuploidy). As a result, loss of chromosomal material (loss of heterozygosity) often occurs, such as *APC* and *p53*. These genes can also be rendered nonfunctional by mutation.

Loss of *APC* function is typically an early event in SCC pathogenesis. The *APC* gene thus has been considered the "gatekeeper" of the colon (Figure 1). Some 85% of all sporadic and inherited colorectal tumors show loss of *APC* function, usually through protein truncation or allelic loss[5]. The *APC* gene is located on chromosome 5q21-q22. The key tumor suppressor function of the *APC* protein lies in its ability to destabilize free β-catenin[6]. Among the 15% of colon carcinomas that retain wild-type *APC*, point mutations have been found in β-catenin that change one of the four serine/threonine residues in the N-terminus, the putative targets of glycogen synthase kinase-3β (GSK-3β)[7]. These mutations thus render β-catenin refractory to phosphorylation by GSK-3β, increasing free β-catenin levels. Accumulation of stabilized free β-catenin is an early event and perhaps the initiating event in intestinal tumorigenesis. Inactivation of both *APC* alleles is found in a majority of small colorectal adenomas in humans and in the smallest detectable tumors in mice heterozygous for an inactivating mutation in *APC*[8]. Furthermore, intestine-specific expression of a dominant-negative form of β-catenin, which lacks the putative GSK-3β targets sites, leads to the development of adenomas[9]. How the loss of *APC* or stabilization of β-catenin leads to development of cancer is not yet fully understood. Once a sporadic adenoma forms, other changes in genetic regulation occur, such as induction of *k-ras* oncogene and loss of function of tumor suppressor genes on chromosome 18q in the region of the deletion in colon cancer (*DCC*) and in pancreatic cancer (*DPC*4) genes. Loss of *p53* gene function occurs late and is believed to be the defining event that drives the adenoma to carcinoma.

Tumors that arise *via* the CIN/tumor suppressor gene pathway are typically microsatellite stable (MSS). The remaining 15% of sporadic CRCs arise through the MSI pathway. The MSI pathway involves the primary loss of function of genes that usually repair DNA base-pair mismatches that occur during the normal process of DNA replication in dividing cells. In humans, at least six different proteins (*hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, *hMSH6*, and *hMLH3*) comprise the mismatch repair system[10]. These proteins form specific heterodimers to coordinate DNA repair and the recruitment of other proteins, such

as polymerases and helicases, necessary for mismatch repair. Germline mutations of DNA mismatch repair genes, predominantly *hMLH1* (33%) and *hMSH2* (31%), are responsible for the familial syndrome of heriditary nonpolyposis colon cancer[11]. In addition, approximately 15% of sporadic tumors from the colon, rectum, and other organs demonstrate MSI[12]. Interestingly, the most common mechanism causing MSI in sporadic colon cancers is not genetic mutation, but rather transcriptional silencing of *hMLH1* as a consequence of methylation of the *hMLH1* promoter[13].

Epigenetic alterations can also contribute to altered gene expression in colon carcinogenesis. The CpG island methylator phenotype occurs when cytosines in the promoter region of genes become extensively methylated. A number of human cancer genes that contain hypermethylation of promoter CpG islands have been identified[14]. These include *hMLH*, $p16^{INK4a}$, and *E-cadherin*. The process of methylation is an area of intense investigation, and it is anticipated that this line of research should help to define further the molecular pathways involved in CRC in a variety of clinical settings.

Molecular features of CRC in IBD

The neoplastic transformation in IBD is thought to be similar to the adenoma-carcinoma sequence in sporadic CRC (Figure 1). However, unlike SCC, where dysplastic lesions arise in one or two focal areas of the colon, in colitic mucosa, it is not unusual for dysplasia or cancer to be multifocal, reflecting a broader "field effect". Many of the molecular alterations responsible for sporadic CRC development also play a role in colitis-associated colon carcinogenesis. The emerging evidence suggests that the two major pathways of CIN and MSI also apply to CACs and with roughly the same frequency (85% CIN, 15% MSI). Distinguishing features of CAC, however, are differences in the timing and frequency of these alterations (Figure 1). For example, *APC* loss of function, considered to be a very common early event in SCC, is much less frequent and usually occurs late in the colitis-associated dysplasia-carcinoma sequence. Conversely, *p53* mutations in sporadic neoplasia usually occur late in the adenoma-carcinoma sequence, whereas in patients with colitis, *p53* mutations occur early and are often detected in mucosa that is non-dysplastic or indefinite for dysplasia.

Methylation is assuming increasing importance as a mechanism contributing to the genetic alterations in CAC (Figure 1). Methylation of CpG islands in several genes seems to precede dysplasia and is more widespread throughout the mucosa of UC patients[15].

CRC risk factors

Several factors have been identified which either increase or decrease CRC risk in the setting of IBD (Table 1). With respect to increasing CRC risk, the most important factor, reproducibly found across many studies, is the duration of colitis. CRC is rarely encountered before 7 years of colitis. The pooled estimate of cumulative CRC incidence in UC in Eaden's meta-analysis was 2% at 10 years, 8% at 20 years, and 18% after 30 years of disease[2]. This may overestimate the risk since many studies in the meta-analysis were from the pre-surveillance era. Thus, a recent study by Rutter *et al* showed lower cumulative incidence of CRC in patients with UC even at the referral-based St. Mark's Hospital in London: 2.5% after 20 years of disease, 7.6% after 30 years, and 10.8% after 40 years of follow-up[16].

Table 1 Factors associated with CRC risk

Factors that increase CRC risk
Longer duration of colitis
Greater extent of colonic involvement
Family history of colorectal cancer
Primary sclerosing cholangitis
Young age of IBD onset (some studies)
Backwash ileitis (possibly)
Severity of histologic inflammation
History of dysplasia
Factors that decrease CRC risk
Prophylactic total proctocolectomy
Regular doctor visits
Surveillance colonoscopy
Chemoprevention

Extent of colitis is an independent risk factor for the development of CRC. The more colonic surface that is involved with colitis, the greater the CRC risk. However, different criteria exist regarding classification of the extent of colitis. Most early reports of surveillance programs used barium enema results at diagnosis as the standard for defining disease extent. A population-based study of over 3000 UC patients in Sweden examined by barium enema demonstrated that patients with proctitis had a standardized incidence ratio (SIR) of 1.7 [95% confidence interval (CI) 0.8-3.2] compared with age-matched population controls without UC, whereas those with left-sided colitis had a SIR 2.8 (95% CI 1.6-4.4), and those with pancolitis had SIR 14.8 (95% CI 11.4 to 18.9)[17]. Endoscopic and histologic evidence of inflammation are valid alternative criteria particularly in high risk patients. Very few studies have correlated CRC risk with histologic extent of disease, even though microscopic evidence of colitis is arguably a better indicator of disease extent than either endoscopic or radiographic changes. Mathy *et al*[18] reviewed 30 colectomy specimens and showed that dysplasia and CRC can arise in areas of microscopic colitis that are proximal to areas of gross colitis, suggesting that indeed histologic changes, even without colonoscopic alterations, might better define disease extent for the purposes of cancer risk. Backwash ileitis, defined as pancolitis with superficial involvement of the terminal ileum, has been suggested as an additional increased risk of CRC[19], but this requires additional confirmation.

IBD patients with a family history of CRC have at least a two-fold higher risk of CRC[20-22]. Both UC and Crohn's disease patients with primary sclerosing cholangitis (PSC) are at particularly high risk for developing colorectal neoplasia[23]. In a population-based Swedish study, the cumulative incidence of CRC in UC patients with PSC was 33% at 20 years[24]. By comparing patients with UC with and without PSC, Shetty *et al*[25] reported an adjusted relative risk (RR) for dysplasia or cancer of 3.15 (95% CI, 1.37 to 7.27) in patients with PSC. Current practice

guidelines recommend all patients with PSC not previously known to have IBD should undergo a colonoscopy to determine their status. For those patients with IBD, screening and subsequent surveillance should begin at the diagnosis of PSC, and if the patient progresses to the point at which liver transplantation is necessary, prophylactic colectomy should be considered. Young age at onset of colitis has been reported to be an independent risk factor for CRC in some[2,17,26], but not all studies[27,28]. There is insufficient evidence to support starting screening and surveillance before 8 years of disease in these patients. The severity of colitis has not been considered an independent risk factor for CRC when activity of disease is defined according to the frequency of clinical exacerbations[22]. However, a case-control study by Rutter *et al* showed that increased severity of inflammation, both endoscopically and histologically, correlates with increased frequency of dysplasia[29]. It is important to realize that the patient with quiescent IBD is also at increased CRC risk.

With regard to reducing CRC risk, there are two main choices: removing the colon versus conducting a lifelong program of surveillance (Table 1). Although prophylactic total proctocolectomy after 7-10 years of colitis would prevent most cancers, it would result in many colectomies that were not necessary and a substantially altered quality of life for patients. Thus cancer prevention in this patient population has focused on periodic surveillance colonoscopies. Surveillance should be viewed as a program that includes regular visits to the doctor, the use of medications to control inflammation (some of which may have chemopreventive effects, see below), and regular colonoscopies. The goal of surveillance colonoscopy is to detect neoplastic lesions before they become biologically dangerous. Thus, the detection and interpretation of dysplasia is crucial to successful surveillance.

DIAGNOSING DYSPLASIA

Macroscopic classification of dysplasia

By definition, dysplasia is unequivocal neoplasia. Despite considerable heterogeneity in appearance[30], dysplasia in IBD is often classified macroscopically as raised or flat, depending on whether it corresponds to an endoscopically visible lesion. Raised lesions, conventionally referred to by the term DALM (dysplasia associated lesion or mass)[13], can appear as polyps, bumps, plaques and velvety patches[31,32]. Such lesions can blend easily with the gross inflammatory abnormalities commonly encountered in colons with IBD, making their endoscopic detection difficult even for experienced practitioners.

Unlike sporadic cancers arising from polypoid lesions, IBD-associated cancers can arise from flat dysplastic lesions. Flat dysplasia is detected microscopically in random biopsies from unremarkable mucosa. Its detection therefore depends on adequate sampling of the mucosa by the endoscopist, or more recently, by chromoendoscopy methods which highlight suspicious lesions and permit targeted biopsies (see below). If random biopsies are performed without dye spray enhancement, it has been estimated that to exclude dysplasia with a 90% certainty, 33 biopsy specimens are required, and to increase the accuracy to 95%, nearly twice the number of biopsy specimens are required[33]. Current surveillance strategies recommend annual colonoscopy with multiple biopsy specimens (4 circumferential) taken from every 10 cm of diseased colon, with additional biopsy specimens at sites of strictures or raised lesions[34]. However, questionnaire surveys have suggested that the number of biopsies taken by endoscopists in routine practice often falls short of recommended guidelines[35,36].

The significance of dysplasia in endoscopically visible lesions came from studies that reported high rates of cancer when patients with such lesions underwent colectomy[31,32]. Blackstone *et al* reported cancers in 7 of 12 DALM-bearing colons, including 5 with only mild or moderate dysplasia in the preoperative biopsies[31]. A subsequent compilation of published results from ten surveillance programs reported cancers in 17 of 40 (43%) colectomies performed because of a DALM[37]. It was concluded that DALM is an indication for colectomy irrespective of the grade of dysplasia in preoperative biopsies. While not fully appreciated at the time, the original studies of DALMs dealt exclusively with lesions that could not be removed endoscopically for microscopic examination. Thus, the significance of such lesions as an indication for surgery is similar to that of endoscopically non-resectable sporadic adenomatous polyps, which frequently harbor invasive cancer at the polyp base despite the presence of low-grade dysplasia in their more biopsy-accessible upper portions.

More recently, we have come to realize that not all types of polypoid dysplasia in patients with IBD carry the same significance. Some polyps may be adenomatous polyps unrelated to colitis and can be managed by endoscopic polypectomy like polyps in the general population[38-40]. One example is the dysplastic polyp encountered in a bowel segment that is entirely free of disease (e.g., in the proximal colon of a patient with left-sided ulcerative colitis). In such cases, one would take the precaution to biopsy the mucosa surrounding the polyp to assure the absence of microscopic disease. Similarly, a dysplastic polyp with a well-defined stalk can be regarded as a sporadic adenoma, even when encountered in a colitic region, if the mucosa lining its stalk is non-dysplastic.

Conservative management is also reasonable for dysplastic polyps that are "adenoma-like"[41,42]. These polyps are endoscopically indistinguishable from sporadic sessile adenomatous polyps, i.e., discrete and ovoid or round, are completely resectable by the endoscope, and are not surrounded by flat dysplasia. Such lesions have long posed a dilemma for endoscopists who were familiar with the DALM concept but reluctant to advocate colectomy for what appeared to be innocuous lesions and possibly nothing more than fortuitous adenomas. Histology has not provided a reliable means of making this distinction in individual cases[43], since the histological features of dysplasia in the setting of IBD and in true adenomas can be virtually identical. A 1999 study from The Mount Sinai Hospital in New York reported that conservative management of a cohort of 48 UC patients with a total of 70 such polyps, including 3 with high-grade dysplasia, resulted in no adverse outcomes during a mean follow-up

period of 4.1 years[41]. Similar conclusions were reached in a concurrent study from Brigham and Women's Hospital[42] that was confirmed upon longer follow-up[44]. As a result, the burden of deciding whether a polyp qualifies as adenoma-like rests with the endoscopist. Molecular markers may ultimately afford a more objective means of making these distinctions[45,46], but to date, these analyses are not applicable to routine clinical practice.

Microscopic classification of dysplasia

Gastrointestinal dysplasia is defined microscopically as replacement of the native intestinal epithelium by an unequivocally neoplastic, but noninvasive, epithelium[47]. It is synonymous with the term "intraepithelial neoplasia" used in other organ systems. A standardized classification system of dysplasia in IBD was established and divided dysplasia into five categories: negative for dysplasia, indefinite for dysplasia, low-grade dysplasia (LGD), high-grade dysplasia (HGD) or invasive cancer[47]. A further subdivision of the indefinite category includes probably negative, probably positive, unknown, however many pathologists regard this as optional.

The cellular abnormalities that define dysplasia in IBD are analogous to those characterizing neoplastic tissue in general, namely nuclear abnormalities reflecting inappropriate cellular proliferation and cytoplasmic abnormalities reflecting clonality and aberrant differentiation. The distinction between low- and high-grade dysplasia depends upon the distribution of nuclei within the cells, low-grade dysplasia being characterized by nuclei that remain confined to the basal half of the cells and high-grade by nuclei that are stratified haphazardly between the basal and apical halves[47]. Not surprisingly, pathologists are frequently confronted with biopsies that lie in a gray zone between the two categories, and some degree of subjectivity is therefore unavoidable. The diagnostic category "indefinite for dysplasia" is an acknowledgement of the difficulty pathologists face in discriminating between dysplasia and reactive epithelial changes, although experienced pathologists are usually able to discriminate between the two.

There is inconsistency among pathologists in the diagnosis of dysplasia on biopsy. In one study[48], there was only 60% agreement for a diagnosis of LGD. Similarly Lim *et al*[49] found that the kappa coefficient for interobserver agreement between ten pairings of five specialist gastrointestinal (GI) pathologists ranged from 0.06 to 0.39. Other studies comparing diagnoses of dysplasia among different pathologists, both prospectively and retrospectively, have concluded that levels of interobserver agreement are fair at best even among specialists in gastrointestinal pathology[50-54]. The best agreement levels tend to occur at the two extremes of negative and high-grade dysplasia and the poorest levels in the two gray zones between low-grade and high-grade dysplasia and on either side of indefinite for dysplasia. From a practical standpoint, it has been recommended that diagnoses carrying serious management implications be reviewed by at least one additional pathologist with expertise in this area[55].

BIOLOGY OF DYSPLASIA

Natural history of dysplasia

The natural history of dysplasia is a key factor contributing to the outcome and success of surveillance. The model shown in Figure 1 suggests that colitic mucosa progresses in a systematic fashion: no dysplasia, indefinite dysplasia, LGD, HGD, and finally invasive cancer. Although this is a useful paradigm that facilitates the study of cancer risk markers in IBD, it remains unclear whether dysplasia of one grade may "progress" (or "regress") to another grade. For example, patients undergoing regular colonoscopic surveillance have developed CRC without any prior dysplasia, and it is not necessary for LGD to progress to HGD before cancer arises in the colon[30,56]. This highlights the need to develop markers that are complementary to dysplasia for predicting CRC risk in IBD patients-a subject of ongoing investigation.

In the meantime, we currently rely upon the histological identification of dysplasia to make management decisions. Refinements in interpreting dysplasia based on the 1983 standardized histological criteria[47] have enabled a more accurate prediction of which patients are more likely to progress to advanced neoplasia by excluding those whose biopsies only show reactive changes secondary to inflammation. This was amply illustrated by the St. Marks group who found that the 5-year cumulative rate of progression from LGD to HGD or cancer rose from 16% to 54% once biopsies were more precisely reclassified[53,57].

Dysplasia (of any grade) is associated with a risk of concurrent CRC in IBD. An early study reported 12 cases, described as unresectable single polypoid masses, collections of polyps, or plaque-like lesions, 7 were found to have adenocarcinoma upon colectomies despite multiple biopsies that had not detected it[31]. In a review of ten prospective surveillance trials, 43% of patients who underwent colectomy because of DALM had coexistent CRC, 42% (10 of 24) patients with HGD, and 16% (3 of 19) patients with LGD who underwent immediate colectomy had synchronous CRC[37]. Ullman and colleagues at The New York Mount Sinai Hospital performed a retrospective cohort analysis of 46 patients with UC who had flat LGD but did not undergo immediate colectomy. They found that 27% (3 of 11) patients who underwent colectomy within 6 months of the initial detection of flat LGD had a surprise finding of cancer or HGD[56]. More recently, Rutter *et al* from the St. Mark's Hospital reported 20% of patients with LGD who proceeded to colectomy had concurrent adenocarcinoma and 39.1% who had follow-up of the LGD progressed to subsequent HGD or CRC[16].

Assuming that early colectomy is not performed, what is the subsequent rate of progression? In the case of patients with HGD, 32% were found to have CRC after some follow-up period[37]. For those with LGD, the probability of eventually progressing to HGD or CRC was 16%-29%[37]. Data from St. Mark's Hospital indicate that the 5-year cumulative probability of progressing from LGD to HGD or cancer is 54%[53]. Strikingly similar results were obtained from The Mount Sinai Hospital, with a 5-year progression rate of 53% among 46 patients

with initial flat LGD[56]. A recent follow-up analysis from the St. Marks' group indicates slightly lower, but still substantial rates of progression[16]. Likewise, a series of 18 patients with LGD followed at the Mayo Clinic demonstrated a 33% 5-year progression rate[58]. Despite these rather similar results from three different patient populations, some authors have reported a substantially lower rate of progression. Befrits *et al* followed 60 patients with flat LGD from the Karolinska Institute in Sweden and found that none developed cancer and only 2 cases of progression to HGD in DALMs over a mean follow-up period of 10 years[59]. In the series by Lim *et al* from Leeds in the U.K., they reported that only 3/29 (10%) patients with LGD progressed to HGD or cancer after 10 years[49]. It is worth noting that in the latter two studies, the designation of LGD included specimens that were interpreted prior to the 1983 consensus guidelines, so they might have included cases with indefinite dysplasia.

Because of uncertainty of flat LGD[56,60], these studies have failed to achieve consensus on proper management of flat LGD. Hence, competing options should be discussed with each patient. A patient confirmed to have multifocal flat LGD (2 or more biopsies with LGD from a single screening or surveillance examination) or repetitive flat LGD (2 or more examinations with at least a single focus of LGD), should be strongly encouraged to undergo prophylactic total proctocolectomy. Furthermore, even for patients with confirmed unifocal LGD (only 1 biopsy positive for LGD in a screening or surveillance examination) should also be offered the option of undergoing prophylactic proctocolectomy, since evidence indicates that a 5-year rate of progression to HGD or CRC in this patient group seems to be similar to that of multifocal LGD[56].

THE MANAGEMENT OF DYSPLASIA

Once a decision is made to place a patient under surveillance, it is recommended that the patient formally agree to enter such a program and is willing to comply. Patients must be made to understand the limitations of surveillance and accept the concept that despite their own cooperation, dysplasia and cancer can still arise even in the hands of skilled endoscopists and pathologists[53].

The best proof that surveillance colonoscopy effectively reduces CRC mortality would be a prospective, randomized, controlled trial in which patients with longstanding IBD would undergo colonoscopic surveillance whereas controls matched for a similar risk profile would not. However, due to ethical, financial and practical limitations, this type of study will likely never come to pass. We must therefore rely on retrospective studies for insights as to the efficacy of surveillance colonoscopy. In a retrospective study by Choi *et al*, patients with chronic UC who developed cancer were divided into those who had surveillance colonoscopy and those who did not. Patients undergoing surveillance colonoscopy were found to have less advanced Dukes' stage CRC than those who did not and correspondingly had an improved 5-year survival rate (77.2% *vs* 36.3%, $P = 0.026$)[61]. However, the best evidence that colonoscopy reduces mortality from CRC in UC comes from case-control studies. In one such study, Karlen and colleagues identified 2 of 40 patients with UC and 18 of 102 controls who had undergone at least one surveillance colonoscopy who died as a result of CRC (RR = 0.29), CRC mortality was reduced by as much as 78%, although this did not reach statistical significance[62]. Another study found a similar degree of protection[22]. In these studies, a protective effect was found for individuals who had even one or two surveillance exams. There is also good evidence from prospective, albeit uncontrolled, studies of surveillance colonoscopy that in general, patients who comply with surveillance have cancers detected at earlier stages compared to those who do not comply[53,63]. Of course, cancers will still arise even within a surveillance program, but in balance, the practice of surveillance is beneficial[64].

Although the gastroenterology community has put its faith in surveillance colonoscopy to prevent CRC in IBD patients, surveillance has its limitations. Previous studies have shown low rates of observer agreement for the histopathologic interpretation of biopsy specimens between general pathologists and GI pathologists, or even among expert GI pathologists[47,48]. Endoscopists fail to take a sufficient number of biopsies to exclude the presence of dysplasia or cancer[33]. Unlike the dysplastic sporadic adenoma which typically assumes a discrete, polypoid shape surrounded by normal mucosa, dysplasia in the colitic colon can be flat or polypoid and is often difficult to discern. This may be particularly troublesome in a colon that is replete with inflammatory pseudopolyps. Furthermore, there is poor understanding of dysplasia amongst trained gastroenterologists. A survey of practicing gastroenterologists and senior GI fellows in the U.S. found that only 19% of respondents correctly identified dysplasia as neoplastic change[35]. Patient drop-out or non-compliance with surveillance contributes importantly to CRC mortality in IBD[49,53] and this must be considered when embarking upon, or continuing, a course of surveillance in individual patients.

RECOMMENDED SURVEILLANCE STRATEGY

Despite the limitations of surveillance colonoscopy, dysplasia remains the best marker for managing cancer risk in IBD. After approximately 7-8 years of colitis, patients should undergo an "initial" surveillance colonoscopy to determine the extent of colitis and check for neoplasia[55]. The entire colon should be examined, with approximately 4 biopsies taken every 10 cm. Some experts suggest taking more biopsies in the distal rectosigmoid (e.g., approximately every 5 cm) since the distribution of neoplasia in UC still shows a distal predominance. Biopsies should be taken from flat mucosa, but if any raised or suspicious lesions are encountered, these should be removed if possible and processed in separate specimen containers (with additional biopsies taken near the base of the polyp). If a patient is experiencing moderate-severe colitis symptoms, one option is to control the inflammation medically prior to performing the examination in order to minimize difficulties with the histological interpretation of

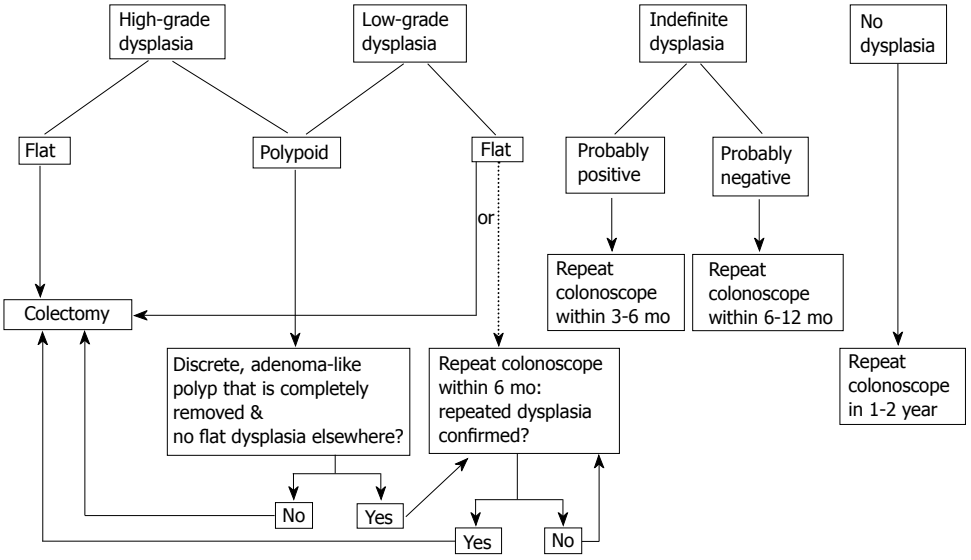


Figure 2 Suggested management scheme for dysplasia. Modified from Ref 71. With permission.

dysplasia. However, one should not defer the colonoscopy too long, since many expert pathologists now feel that they can readily interpret dysplasia even in the presence of active inflammation.

Figure 2 depicts a recommended surveillance strategy. If no dysplasia is detected, the examination should be repeated in 1-2 years. This interval derives in part from studies reporting that interval cancers can develop within 2 years after a surveillance examination[53]. If indefinite dysplasia is reported, the nature of the uncertainty should be discussed with the pathologist. If the suspicion of dysplasia is high (i.e., probably positive), repeat biopsy within 3-6 mo or less may be indicated; if low, the interval can be lengthened to every 6-12 mo. If LGD is detected in a discrete polyp that can be readily resected endoscopically and there is no flat dysplasia immediately adjacent to the polyp or elsewhere in the colon, surveillance can be continued, although the frequency of examinations can be temporarily reduced to every 3-6 mo, particularly to re-evaluate the area of polypectomy. Tattoo of the polypectomy site is advised to permit relocalization of the area on subsequent exams. If LGD is detected in flat mucosa (whether unifocal or multifocal), and is confirmed by a second expert GI pathologist, colectomy should be strongly considered. If the patient refuses, repeat surveillance exams should be undertaken within 3-6 mo or less. However, the patient should be advised that a negative subsequent examination is no assurance of safety, and that temporizing until there is histological progression to HGD or cancer as the indication for colectomy is risky. A patient in whom flat HGD or adenocarcinoma is found and confirmed by two expert pathologists should undergo colectomy unless serious co-morbidities dictate otherwise. If HGD is diagnosed in an adenoma-like polyp but it is completely removed without evidence of flat dysplasia in the adjacent mucosa or elsewhere in the colon, continued surveillance can be entertained. As with any set of recommendations, decisions should be individualized according to the situation of the patient. Hopefully, strategies for surveillance will become more refined as more knowledge of the natural history of dysplasia is obtained.

Patients who have only had small intestinal Crohn's disease without colonic involvement are not considered to be at high risk for CRC. For patients with Crohn's colitis, much less is known. To date, only one practice-based retrospective surveillance study has been reported in patients with Crohn's colitis[65]. Of 259 patients with Crohn's colitis affecting at least one-third of the colon for at least 8 years, 16% were found to have dysplasia or cancer over a 16 year period in which 663 examinations were performed, and there were no cancer deaths. While we await additional data on the subject, it seems wise to follow a UC-based surveillance strategy for patients with at least 8 years of substantial Crohn's colitis. An important question that remains to be clarified is whether patients with dysplasia or cancer in the setting of segmental Crohn's colitis can undergo segmental resection of the involved area or should proceed to a more extensive UC-like surgical approach. Another dilemma that is encountered with Crohn's colitis is the management of strictures. This occurs more so than with UC. Finding a stricture in a colon of a patient with UC usually means an underlying malignancy, especially if the stricture is causing symptoms, and is located in the proximal colon[66]. However, since most strictures in Crohn's colitis are benign, the patient can often be managed conservatively. Surveillance of such patients often requires using a narrower colonoscope or sometimes dilating a stricture to visualize the proximal mucosa[65]. Consideration should be given to adding brush cytology of strictures to regular forceps biopsies, and performing a barium enema to evaluate for colonic wall irregularity.

New endoscopic techniques and molecular screening approaches

Recent publications on chromoendoscopy have demonstrated a greater yield for dye-spray targeted biopsies compared with numerous non-targeted biopsies and thus enhance the endoscopic detection of dysplastic lesions in colitic colons. In a randomized trial by Kiesslich *et al*[67], intraepithelial neoplasia was more than three times as likely to be detected using chromoendoscopy compared with surveillance using nontargeted biopsies (32/84 compared

with 10/81; $P = 0.003$). Rutter *et al* detected no dysplasia in 2904 non-targeted biopsies in 100 patients, but in targeted biopsies, nine dysplastic lesions were detected, seven of which were visible only with dye spraying by using indigo carmine instead of methylene blue[68]. A recent report by Ochsenkuhn *et al* from Munich, Germany showed a low frequency of colorectal dysplasia in patients with long-standing IBD by fluorescence colonoscopy with 5-aminolevulinic acid[69].

Other approaches worth mentioning are to examine the biopsy tissue of patients with IBD for molecular alterations. The best tested of these are aneuploidy, mutations in p53 and ras, and glycosylation abnormalities, particularly increased expression of sialyl Tn antigen (sialyl 2,6 N-acetylgalactosamine)[70]. Because the DNA shed into stool should theoretically provide a more comprehensive sampling of abnormal cells than random pinch biopsies, stool DNA testing could potentially contribute to the management of patients with long-standing IBD who are at risk for developing CRC.

Chemoprevention

Despite the relative protection afforded by surveillance colonoscopies in IBD, there are still patients who develop CRC despite seemingly optimal surveillance. This raises the issue of whether chemoprevention in the form of either medications or dietary supplements might help reduce the risk of CRC in IBD[71].

Aspirin and other NSAIDs markedly reduce the incidence of, and mortality from, sporadic CRC. Since many patients with IBD take NSAIDs in the form of 5-aminosalicylates (5-ASA), investigators have asked whether 5-ASA compounds might also be protective. Although no study to date has been performed in a prospective manner specifically to address this question, the available data suggest that this may be so[72]. If 5-ASA compounds prevent colonic neoplasia by suppressing inflammation, it follows that other anti-inflammatory medications used in IBD patients should also be protective against CRC. Although one study reported that the use of systemic steroids, and even topical steroids resulted in a significant CRC risk reduction[22], and others confirm this observation[73], steroids cannot be used long-term for chemoprevention. There appears to be no chemopreventive activity of 6-mercaptopurine or azathioprine[74].

In the setting of sporadic CRC, low folate intake has been associated with an increased risk for developing colorectal adenomas and carcinomas[75,76]. Patients with chronic IBD are predisposed towards folate deficiency because of inadequate nutritional intake, excessive intestinal losses with active disease, and reduced intestinal absorption from competitive inhibition from sulfasalazine use. Results of two studies suggest a trend towards protection against CRC in folate users, although neither study demonstrated statistical significance[77,78]. Nonetheless, since it is rather safe and inexpensive, folate supplementation should be considered for CRC risk reduction in patients with longstanding IBD.

In animal models of colon carcinogenesis, ursodiol inhibits carcinogenesis-an effect that may be due to the ursodiol reducing the colonic concentration of the secondary bile acid deoxycholic acid. Ursodiol also has anti-oxidant activity. A study of UC patients with PSC demonstrated that ursodiol use was strongly associated with decreased prevalence of colonic dysplasia[79]. This protective effect remained after adjusting for duration of colitis, age at onset of colitis, and sulfasalazine use. In a follow-up to the randomized, placebo-controlled trial by Pardi *et al* at the Mayo Clinic, 52 patients with chronic PSC and chronic UC (mean 13 years) were followed for a total of 335 person-years. Ursodiol use was associated with a significant protection against the development of dysplasia and cancer (RR = 0.26, $P = 0.034$)[80]. At the present time, however, we do not know whether ursodiol can prevent neoplastic progression in UC patients without PSC.

Recently, there has been interest in the role statins may play as chemopreventive agents in a variety of cancers. In a population-based case-control study of patients who had diagnosis of CRC in northern Israel between 1998-2004, statin therapy was associated with a modest reduction in CRC in the non-IBD population, but a substantial 94% risk reduction in patients with IBD was observed in a subset analysis of a small number of patients[81]. Further studies will need to verify this benefit.

Although a single retrospective cohort study suggests that therapy with 6-mercaptopurine is not chemopreventive (or carcinogenic), there remain insufficient data regarding the chemopreventive role of immunomodulators in order to make recommendations and likewise, whether patients who require immunomodulator therapy should continue their 5-ASA therapies[74].

Small bowel cancer in IBD

Most cancers of the small bowel in Crohn's disease are adenocarcinoma, usually in the terminal ileum or jejunum. The most common clinical presentation of small bowel cancer is intestinal obstruction[82]. Other important symptoms include diarrhea, weight loss, and abdominal fistulae. These symptoms are also found in Crohn's disease. Risk factors for developing carcinoma in small bowel segments of involved mucosa in patients with Crohn's disease are poorly defined, although case reports document them in strictured mucosa and fistulae[83-85]. Surgery should be considered if the fistulae or stricture cannot be adequately examined, or symptoms substantially worsen. There is some suggestion that 5-ASA compounds might lower the risk of small intestinal adenocarcinoma[86].

A number of studies have demonstrated an increased risk of developing adenocarcinoma of the small intestine with small intestinal Crohn's disease. Although the absolute number of cases of small bowel adenocarcinoma is low, because of the rarity of this cancer in the general population, the risk is approximately 10-12-fold greater than the general population[4,86]. In the Uppsala study[87], the investigators reported only one observed case compared with 0.3 expected cases, but the confidence interval was wide. In the Copenhagen study[88], two cases were observed *vs* 0.04 expected cases, a 50-fold increased occurrence. In the Tel Aviv study[89], none of the patients developed small cancer. The material was probably far too small to expect any small bowel cancer cases. In Oxford, a 10-fold increased relative risk was observed for cancer of the small

intestine[90]. A population-based Swedish study revealed a significantly increased number of cancer of the small intestine (standardized morbidity ratio, 15.64; 95% CI, 4.26-40.06), however, the occurrence of colorectal cancer was not increased[91]. Another population-based Canadian study encompassing the years 1984-1997 demonstrated an increased incidence rate ratio of carcinoma of the small intestine (17.4; 95% CI, 4.16-72.9)[4].

Patients with UC who have undergone total proctocolectomy with ileal pouch anal-anastomosis (IPAA) have a very small risk of dysplasia arising within the ileal mucosa of the pouch itself. The risk is thought to be higher in patients with chronic pouchitis and associated severe villous atrophy[92], but this has not been shown in all series[93]. Indeed, a study of 160 patients who underwent biopsy a total of 222 times with an average surveillance time of 8.4 years after surgery showed that in 1800 pouch-years of surveillance, only one patient had focal LGD of the pouch[94]. The risk of neoplasia is greater in the anal transitional mucosa between the pouch and the anal canal, particularly if a cuff of rectal mucosa has been left, and if the indication for the IPAA was rectal dysplasia or cancer[95]. While there are currently no guidelines for endoscopic surveillance after an IPAA procedure, in those patients who have chronic pouchitis and severe villous atrophy or whose original indication for IPAA was dysplasia or cancer, a program of periodic endoscopy with biopsies, paying particular attention to any anal transition zone, is reasonable.

Other cancers in patients with IBD

Squamous cell carcinoma of the anus has been reported in patients with longstanding, complicated perianal Crohn's disease[85]. Worsening perianal symptoms in such patients should warrant heightened vigilance for this tumor which often requires examination under anesthesia for adequate tissue diagnosis.

An increased risk for hepatobiliary cancers in patients with UC has been found in several[4,96-98] but not all[87] studies. For many of these patients, primary sclerosing cholangitis was the predisposing factor.

The risk of hematopoietic cancer in patients with IBD has been a growing concern. Early case series from The Cleveland Clinic[96] and The Mount Sinai Hospital[99], as well as other centers[100] reported an increase in leukemia in patients with UC. A recent large cohort study from Sweden[101], which included nearly 50 000 IBD patients concluded this population has a marginally increased risk of hematopoietic cancer, and in UC, lymphoma occurred as expected (SIR 1.0) but myeloid leukemia occurred significantly more often than expected (SIR 1.8). In Crohn's disease, there was a borderline significant increased lymphoma risk (SIR 1.3), essentially confined to the first years of follow up. However, population-based studies from Denmark[97], Sweden[87] and Canada[4] have failed to substantiate any increased risk of leukemia. Likewise, although an increased number of lymphomas have been reported in some case series[99,102], other series[96] and several population-based studies[87,91,97,98] do not support the notion that patients with UC or Crohn's disease are at increased risk of lymphoma. However, a population-based study from Canada reported an increased rate of lymphoma among male patients with Crohn's disease[4].

The risk of lymphoma or leukemia in IBD has raised concerns regarding the lymphogenic potential of immunomodulatory therapy. Following the introduction of tumor necrosis factor inhibitors in the treatment of Crohn's disease, subsequent reports indicated an excess of malignant lymphoma among treated patients[103,104] and raised fears of an iatrogenic lymphoma risk. However, these reports have also highlighted the lack of robust data on the expected occurrence of malignant lymphomas in TNF naïve (but otherwise treated) patients with IBD[105-107]. Studies examining the risk of lymphoma associated with azathioprine (AZA) and 6-mercaptopurine (6-MP) have yielded variable results. Heterogeneity in the type, dose, and duration of immunomodulatory therapy may be responsible for this discrepancy. A few studies with suboptimal dosing failed to demonstrate an increased risk of lymphoma[4,108-112]. In contrast to these reports, other studies have demonstrated an increased risk of lymphoma after purine analog therapy[113-115]. In one such study, Kandiel *et al* reported a 4-fold-higher risk of lymphoma inpatients treated with AZA or 6-MP compared with the general population[114]. Another recent study showed a statistically significant increase in the development of malignancies among IBD patients treated with 6-MP who developed sustained leucopenia[115].

FUTURE DIRECTIONS

The future looks promising with respect to new developments in the management of cancer risk in IBD. Chromoendoscopy is likely to be used more for management, but whether the predictable increase in the yield of dysplasia will alter the overall natural history remains to be studied. In the modern era of molecular diagnostics, tissue and even stool samples of patients with IBD can be investigated for molecular alterations. For example, University of Washington investigators have demonstrated that because there is often widespread genomic instability throughout the colon of IBD patients, it may be possible to analyze rectal biopsies by DNA fingerprinting or fluorescence *in situ* hybridization methods[116] to identify patients at particularly high risk. The advent of technology to extract human DNA from stool and look for specific DNA mutations associated with sporadic colon carcinogenesis[117,118] implies that a similar approach may also be worthwhile in IBD patients. It is anticipated that refinements in our knowledge of cancer biology, clinical practice, and molecular discovery will bring a new level of sophistication to the management of patients with longstanding IBD and lower the incidence of CRC in this high-risk population.

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TOPIC HIGHLIGHT

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Immunopathogenesis of inflammatory bowel disease

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Abstract

Crohn's disease and ulcerative colitis are chronic relapsing immune mediated disorders that results from an aberrant response to gut luminal antigen in genetically susceptible host. The adaptive immune response that is then triggered was widely considered to be a T-helper-1 mediated condition in Crohn's disease and T-helper-2 mediated condition in ulcerative colitis. Recent studies in animal models, genome wide association, and basic science has provided important insights in in the immunopathogenesis of inflammatory bowel disease, one of which was the characterization of the interleukin-23/Th-17 axis.

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Key words: Crohn's disease; Ulcerative colitis; Innate and adaptive immune system

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INTRODUCTION

Inflammatory bowel disease (IBD) results from a complex series of interactions between susceptibility genes, the environment, and the immune system. The clinical features of the disease, histopathological findings, and the therapeutic efficacy of immunosuppressive drugs indicate an involvement of the immune system in the pathogenesis of the disease. Various components of the mucosal immune system are implicated in the pathogenesis of IBD. These components include luminal antigens, intestinal epithelial cells (IECs), cells of the innate and adaptive immune system, and their secreted mediators. An overview of the working hypothesis of IBD is depicted in Figure 1. Either a mucosal susceptibility or defect in sampling of gut luminal antigen leads to activation of innate immune response, possibly mediated by enhanced Toll-like receptor (TLR) activity. The stimulated dendritic cells (DC) then mediate the differentiation of naïve T-cells into effector T-cells. Crohn's disease (CD) is a predominately Th1 and Th17 mediated process, while ulcerative colitis (UC) appears to be predominately mediated through Th2 and NK T-cells. In this review, the effects of these components in the immunopathogenesis of IBD will be discussed.

BACTERIA AND THEIR ASSOCIATED MOLECULES IN THE INTESTINE

The distal ileum and colon contain high concentrations of bacteria (> 10^{12} organisms/g). These may include pathogens that could be directly responsible for initiating and promoting IBD in the context of an underlying genetic mucosal or immune defect. Studies have shown that there are differences in the microbiota between healthy and IBD subjects (Table 1). One of the differences is that there is a decrease biodiversity in IBD compared to healthy subjects by 30%-50%[1]. The reduction in diversity in inflammatory bowel disease was due to loss of normal anaerobic bacteria such as *Bacteroides*, *Eubacterium*, and *Lactobacillus* species[1]. Diversity is generally thought to be desirable by conferring resiliency to an ecosystem[2]. For example diversity would provide functional redundancy in the microbial community to ensure that key processes such as breaking down nutrients and preventing random chaotic fluctuation of bacterial subpopulations. Restriction of biodiversity in the human gut may lead to dysbiosis and result in mucosal insult. A second difference is that there are fewer *Firmicutes* in IBD compared to healthy subjects; 13 distinct *Firmicutes* ribotypes were identified in CD microbiota compared to 43 in healthy microbiota ($P < 0.025$)[3]. *Firmicutes* are Gram-positive class of bacteria that include the genus *Clostridium* and *Bacillus*. Third, there are pathogens that are found in increasing frequency in IBD and have been implicated to associate with its development. These pathogens include *Pectinatus*, *Sutterella*, *Fusobacterium*, *Verrucomicrobium*, various *Clostrodia*, *Mycobacterium paratuberculosis*, *M. paramyxovirus*, Listeria monocytogenes, and Helicobacter hapaticus[4,5]. Despite the differences in the microbiota, one must keep in mind that the dysbiosis seen in IBD patients may not be causal, but simply reflect the different ecological conditions of the inflamed gut such as changes in pH,

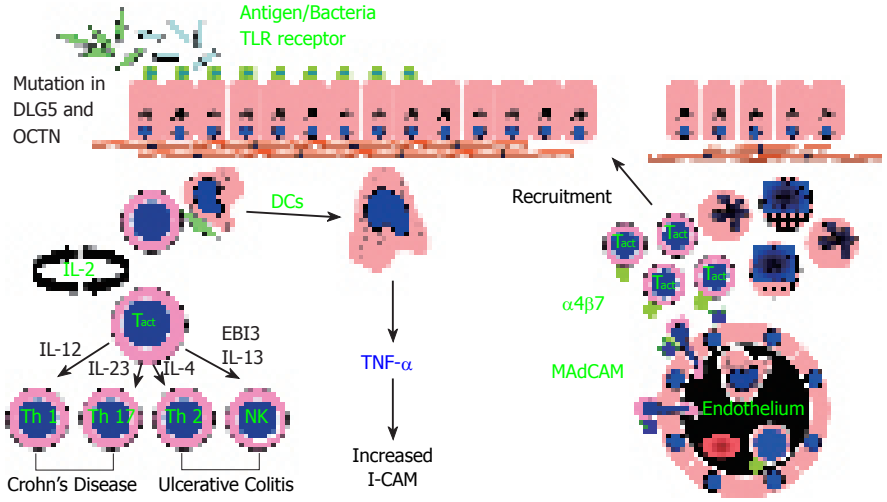


Figure 1 Working hypothesis of inflammatory bowel disease. A defect occurs in sampling of gut luminal antigen, possibly mediated by enhanced Toll-like receptor activity and controlled by other genetic factors (mutations in DLG5 and OCTN). Over-response to antigens results in stimulated dendritic cells (DC) that recruits and generates various T-cell subtypes, which then initiate a cascade of immunologic events leading to mucosal inflammation. Adhesion molecules such as intercellular cell adhesion molecule 1 (ICAM1) are important for circulating mononuclear and polymorphonuclear cells to adhere and migrate to the inflamed gut mucosa. Crohn's disease (CD) is a predominately Th1 and Th17 mediated process, while ulcerative colitis (UC) appears to be predominately mediated through Th2 and NK T-cells.

Table 1 Comparison of microbiota between healthy and IBD subjects

Healthy subjects	IBD subjects
High biodiversity	Low biodiversity
Stable microbiota	Dysbiosis
Increased gut commensals	Increased gut pathogens
Higher firmicutes	Lower firmicutes

redox potential, substrate availability, *etc.*

The importance of the microflora in the induction of and maintenance of disease has been demonstrated in murine model of colitis. For example, mice that are deficient in the cytokines IL-2 or IL-10 or rats containing the *HLA-B27* transgene develop IBD in the presence of a normal microflora but not in a sterile germ-free environment[6-8]. Moreover, experimental colitis is attenuated when animals are treated with broad spectrum antibiotics[9]. Different bacteria may lead to different types of colitis in the same genetic host. In *IL-10*$^{-/-}$ mice, *E. coli* induced proximal colitis whereas *Enterococcus faecalis* lead to distal colitis[10]. Indirect evidence suggesting a role of the intestinal microbiota in IBD came from a double blinded, randomized controlled trials that showed imidazole antibiotics decrease the risk of post-operative recurrence of CD[11].

The surfaces of microorganisms typically bear repeating pattern of molecular structures as do their nucleotides. These motifs are designated as Pathogen Associated Molecular Patterns (PAMP). Some of the PAMPs include complex macromolecules such as lipopolysaccharide (LPS), peptidoglycan (PGN), polypeptides (flagellin), and nucleic acid (CpG rich DNA). Receptors of the PAMPs are called pattern recognition receptors (PRR). Toll like receptors (TLR) are examples of PRR. TLR recognition usually triggers the innate immune system, leading to an inflammatory response. Interaction between PAMPs and PRR are illustrated by the CpG dinucleotides activation of innate immunity *via* TLR9 (Figure 2). Oligonucleotides containing CpG motifs (CpG-ODN) prevented lesions and reduced the release of inflammatory cytokines when given before the DSS-induced colitis[12-14]. However, if CpG-ODN is given after DSS-colitis induction, the colitis is worsened[13-15]. In TLR9-deficient mice, the onset of DSS-induced colitis was not prevented, but chronic inflammation was reduced[12,15]. This is due to the dual anti-inflammatory effect of TLR9 signaling that is mediated through type I interferon (IFN)[16].

The study of autoantibody has provided evidence that immunologic responses to bacterial products is involved in the induction of inflammatory bowel disease. Reactivity to bacterial antigens was initially shown by findings of modest increase in serum antibodies to 7 bacterial pathogens in a group of CD patients[17]. Subsequently, several studies showed serum responses to various bacterial antigens and loss of tolerance to pathogenic as well as commensal bacteria in clones derived from peripheral and lamina propria T-cells from CD subjects[18-22]. This indicates that disordered features of T-cell microbial recognition and effector function are likely to be important to IBD disease biology.

Antibodies to specific bacterial antigens are clustered into 4 major groups in IBD patients: (1) antibody responses against oligomannan [anti-Saccharomyces cerevisiae (ASCA)][23]; (2) antibody responses to both *Escherichia coli* outer membrane protein C (anti-OmpC) and a CD-related protein from *Pseudomonas fluorescens* (anti-CD-related bacterial sequence)[24,25]; (3) antibody response to nuclear antigens [perinuclear antinutrophil cytoplasmic antibody (pANCA)][26]; or (4) antibody response to the flagellin, CBir1[27]. These antibodies provides immunophenotypic associations to distinguish between UC and CD. CD patients with high levels of IgG and IgA ASCA and the absence of pANCA have more aggressive, small bowel fistulizing and fibrosing disease, and patients with high-level pANCA, in the absence of ASCA, have an ulcerative colitis-like colonic disease[28-30]. In a prospective study, pANCA expression is associated with development of pouchitis after ileal pouch-anal anastomosis (IPAA)[30]. The site of pANCA production has been localized to the gastrointestinal mucosa and was not found in detectable amounts in the periphery, suggesting the importance of mucosal immune response to luminal antigen[31]. In fact, absorption of either human or mouse pANCA-positive sera with enteric bacterial antigens greatly reduced or abolished the specific perinuclear staining of pANCA,

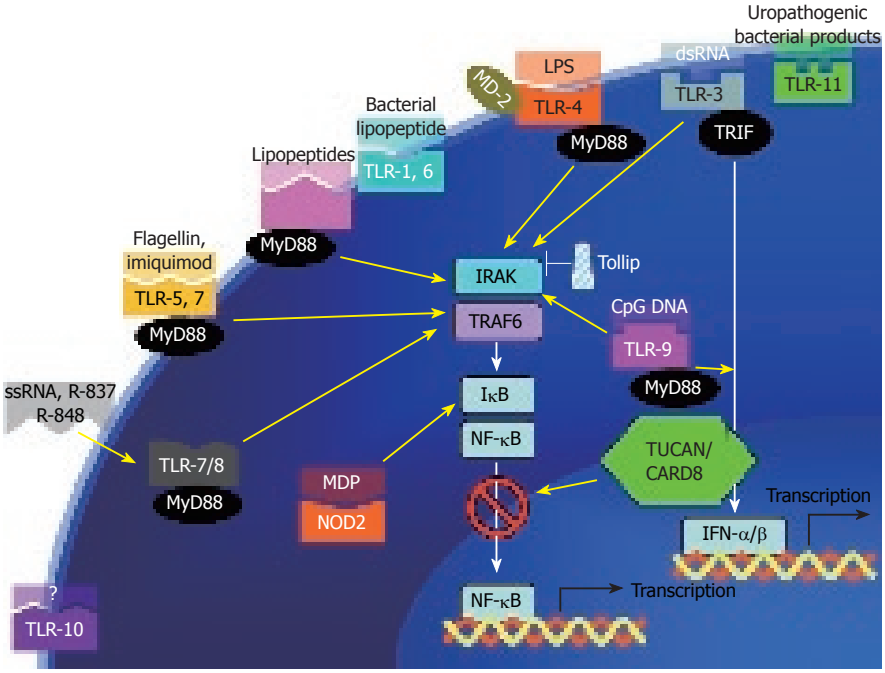


Figure 2 PRRs and their corresponding ligands. Toll-like receptors on the cell membrane (TLR-1, -6, -10, and -11) and intracellular (TLR-7, -8, and -9) selectively bind to various bacterial, viral, or fungal components. A major convergent pathway is through myeloid differentiation primary response protein MyD88, which activates NF-κB. The death domain of MyD88 then recruits downstream IL-1 receptor-associated kinase (IRAK) to the receptor complex. IRAK is then autophosphorylated and in turn recruits TNF receptor-associated factor 6 (TRAF6). TRAF6 then activates kinases including NF-κB-inducing kinase (NIK) and mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1). Inhibitor of NF-κB degradation (IκB) is subsequently phosphorylated and degraded, resulting in NF-κB nuclear translocation. NF-κB then activate genes involved in inflammatory response including IL-1b, TNF, IL-6, IL-8, and ICAM1. Toll-inhibitory protein (Tollip) is one of the negative regulators of the innate immunity. Activation of type I IFN (IFN-α/β) also has anti-inflammatory function in colitis.

indicating that pANCA seroreactivity reflects a cross-reactivity to enteric bacterial antigens[32].

The flagellin, CBir1, has been identified as an immunodominant antigen that is capable of inducing severe colitis in mice and eliciting antibody responses in approximately 50% of CD patients[33]. In contrast, patients with UC or other inflammatory gastrointestinal diseases and control subjects have little or no reactivity to CBir1[33]. Serum responses to CBir1 independently identify a subset of patients with small-bowel, internal-penetrating, and fibrostenosing CD that have no correlation to levels of ASCA, OmpC, and pANCA[23]. Molecular studies in CBir have also shed light on the underlying immunopathophysiologic mechanisms related to this antigenic response. CBir involves both the innate and the adaptive immune system. CBir flagellin is found to be the ligand for TLR5 (Figure 2)[34]. Bacterial flagellin is a structural protein that makes up flagella, which functions in bacterial chemotaxis and adhesion of host tissues. TLR5 is expressed on the basolateral surface of intestinal epithelial cells and hypothesized to be important in the recognition of invasive flagellated bacteria at the mucosal surface. TLR5-flagellin interaction leads to the activation of the transcription factor NF-κB, resulting in the transcriptional induction of proinflammatory cytokines and maturation of human dendritic cells (Figure 1)[35,36]. The expression of antibodies to CBir1 and transferring of a CBir1-specific CD4 + Th1 cells to induce colitis in mice is indicative of an adaptive immune response to this antigen[27].

These results show that some endogenous micro-organisms provide antigenic stimulation through PAMP activation of PRR to maintain intestinal homeostasis: either inflammation through the release of inflammatory cytokines or mucosal protection through type I IFN. In addition, serum reactivity to bacteria and their associated molecules may indicate a predisposition to certain mucosal antigen that could reflect the clinical manifestation and type of IBD. These distinct antibody response patterns to either UC or CD indicate unique pathophysiologic mechanisms to IBD.

INTESTINAL EPITHELIAL CELLS

The 400-mm^2 single layer of gut epithelial cells is the primary cellular barrier to prevent antigens from encountering the immune system. The gut maintains an extensive and active gut-associated lymphoid tissue (GALT). Peyer's patches (PP) are aggregates of lymphoid tissue that are interspersed at intervals just beneath the gut epithelium. They are comprised of B-cell follicles located under specialized areas, or 'domes,' of epithelium known as follicular-associated epithelium (FAE), with T-cell zones occupy the areas between follicles[37]. FAE contains cells called multi-fenestrated or M cells whose function is to transport luminal antigen into the dome area of the follicle[37]. Dendritic cells (DCs) are antigen presenting cells that act as sentinels, sending processes between gut epithelial cells and sample commensal and pathogenic bacteria[38]. Defects in the epithelial barrier may allow GALT to be exposed to excess or harmful luminal antigens, resulting in the production of pro-inflammatory cytokines such as TNF-α and mucosal inflammation.

Tight junction between epithelial cells allows for the selective entry of fluids, nutrients, and micro-organisms. Normal gut permeability is dependent on an intact epithelium, surface mucus, peristalsis, and the secretion of host protective factors. Alterations in gut permeability factors have been reported. Two genes that are associated with Crohn's disease which affect mucosal permeability are the IBD 5 gene organic cation transporter (*OCTN*) and guanylate kinase *DLG5* (Figure 1). Missense mutations in the *OCTN* gene form a haplotype that is associated with susceptibility to Crohn's disease and the mutant protein has impaired ability to pump xenobotics and amino acids across cell membrane[39]. The other protein, *DLG5*, is a scaffold protein involved in the maintenance of

epithelial integrity. Two distinct haplotypes in the *DLG5* gene represented by nonsynonymous single-nucleotide polymorphism in the gene are associated with IBD[40]. It is proposed that the mutation probably impedes scaffolding of *DLG5* and thus, affect epithelial polarity[40]. Altered mucus production has also been found in IBD patients with thinner than normal colonic mucus layer in UC and a thicker than normal layer in CD[41]. Several studies have shown that the there is an increased number of adherent bacteria in IBD patients[42-44]. This relationship holds true in both the mucus layer and at the epithelial surface where bacteria are less abundant. It is possible that the disruption of the function of *OCTN* and *DLG5* and abnormal mucus composition alters the gut permeability. Altered gut permeability may lead to increased bacteria adherence and inappropriate exposure of the mucosal immune system to bacterial products causing inflammation.

INNATE IMMUNITY

Studies described above indicate that microbial structures can cause gut mucosal inflammation by involving the innate immune system. Adhesion molecules such as intercellular cell adhesion molecule 1 (ICAM1) are important for circulating mononuclear and polymorphonuclear cells to adhere and migrate to the inflamed gut mucosa (Figure 1)[45]. Proinflammatory molecules are preferentially produced by innate immune cells that have migrated to the inflammatory mucosa owing to increased expression of PRR such as TLR[46]. Antigen-presenting cells such as dendritic cells (DCs) and macrophages possess TLRs with different specificities for microbial products and induce innate immune response. Immunopathogenic role of TLR signaling in IBD is in the process of being elucidated.

NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN (NOD) PROTEINS

NOD proteins are a distinct subset of PRRs that have important roles in innate immunity as cytoplasmic sensors of microbial components, allowing for regulation of inflammatory processes and apoptosis[47]. The importance of these PRRs is highlighted by the fact that mutations in the gene that encodes NOD2 occur in a subset of patients with CD and polymorphisms in the gene that encodes NOD1 are associated with IBD[48-50]. The NOD1 protein (encoded by caspase-recruitment domain 4 gene, *CARD4*) and NOD2 protein (encoded by CARD15) are expressed by antigen-presenting cells (APCs) and epithelial cells[47]. NOD2 is a general sensor of most bacteria because it recognizes muramyl dipeptide (MDP), which is a component of both Gram-positive and Gram-negative bacteria[51]. In contrast, NOD1 senses mostly Gram-negative bacteria because the ligand for NOD1, peptidoglycan (PGN) derived peptide γ-D-glutamyl-mesodiamino-pimelic acid (iE-DAP), is not present in Gram-positive bacteria except for *Listeria* and *Bacillus spp*[52]. The main outcomes of NOD1 and NOD2 activation is the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathway (Figure 2)[51,53-55]. In addition, activation of NOD1 by its ligands leads to the activation of JUN N-terminal kinase (JNK)[47]. Activations of NF-κB, MAPK, and JNK pathways lead to the production of pro-inflammatory mediators[47].

There are 3 models of how *CARD15* mutations are associated with CD, however, studies have shown inconsistencies in these models. The first view is based on the study that showed NOD2 is a negative regulator of IL-12 mediated by PGN[53]. Loss-of-function mutation in CARD15 results in an excessive NF-κB dependent IL-12 response by splenic macrophages to create a milieu that supports Th1 cell mediated colitis. This is supported by findings that production of IL-12p70 by peripheral blood monocytes derived dendritic cells in response to PGN is higher in patients with CARD15 mutation than in normal subjects[56]. In contrast to the study by Watanabe *et al*, another study that used bone-marrow derived macrophages did not result in enhanced proinflammatory cytokine production[55]. Other studies show that CARD15 mutation results in reduced anti-inflammatory cytokine responses (IL-10 and TGF-β) to several TLR ligands[57,58]. These indicate that NOD2 signaling should augment an anti-inflammatory response and not inhibition of TLR responses in normal individuals. However, defective anti-inflammatory response secondary to CARD15 mutation does not sufficient explain increased cytokine production that is characteristic of CD.

The second model suggested that a loss-of-function NOD2 lead to susceptibility to CD as a consequence of impaired host defense. This may be due to impaired NOD2-dependent α-defensin production by Paneth cells, and thus, lead to impaired control of pathogenic bacteria and inflammatory condition[59]. This is also supported by the finding that CD patients with CARD15 mutation have a more pronounced defective production of a-defensin-5 and -6[60]. Consistent with the impaired mucosal host defense, NOD2 deficient mice developed worse liver infection to *L. monocytogenes* when the pathogen was given orally compared with systemic administration[55]. However, there are several studies that did not support the idea that CD is due to impaired host defense as a consequence of NOD2 mutation. One of the strongest argument is the data that mice in with ablation Paneth-cell population or mice that lacks α-defensins develop evidence of acute or chronic inflammation[61,62]. In addition, NOD2-deficient mice that were administered *L. monocytogenes* did not cause mucosal infection[55].

The third model proposes gain-of-function mutations in the *CARD15* gene that are associated with CD. This possibility is supported by studies in mice carrying a mutation that is analogous to the most common CD susceptibility allele, 3020insC[63]. The mutant mice show increased NF-κB activation and enhanced production of IL-1β upon MDP stimulation of bone marrow-derived macrophages[63]. Inconsistent with the above study, peripheral-blood mononuclear cells isolated from CD patients that carrie the 3020insC allele show a defect in IL-1β secretion[64]. Additionally, epithelial cells transfected with 3020insC CARD15 mutation have defective NF-κB activation upon MDP stimulation[51,65].

There are several views and contradictory findings on the role of NOD protein in the pathogenesis of IBD.

Among the unanswered questions are whether CARD15 mutations confer a gain- or loss-of protein function. Perhaps the mutations have different effects in different context, i.e. in the APC *versus* epithelial cells or in the mucosa *versus* the periphery. More studies are needed to elucidate the immunopathogenesis of CARD15 mutations in IBD.

TOLL LIKE RECEPTOR AND ACTIVATION OF INNATE IMMUNE RESPONSE

Eleven TLRs have been identified which are characterized by three common structural features: (1) divergent ligand binding extracellular domain with leucine rich repeats; (2) short transmembrane region; (3) a highly homologous cytoplasmic Toll/interleukin 1 receptor (TIR) domain. Even though each type of TLR engages a different PAMPs, a major convergent pathway is through myeloid differentiation primary response protein MyD88, which activates NF-κB. Upon activation, TLRs form homodimers leading to a conformation change in the cytoplasmic TIR domain and recruitment of MyD88[66]. The death domain of MyD88 then recruits downstream IL-1 receptor-associated kinase (IRAK) to the receptor complex[66]. IRAK is then autophosphorylated and in turn recruits TNF receptor-associated factor 6 (TRAF6). TRAF6 then activates kinases including NF-κB-inducing kinase (NIK) and mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1)[67]. Inhibitor of NF-κB degradation (IκB) is subsequently phosphorylated and degraded, resulting in nuclear translocation of NF-κB[66]. NF-κB belongs to Rel family of DNA binding transcription factor that bind characteristic sequence motifs and activate genes involved in immune and inflammatory response[66]. This is illustrated in Figure 2.

Activation of NFκB stimulates expression of numerous molecules relevant to the pathogenesis of IBD including factors involved in the inflammatory response (IL-1b, TNF, IL-6, IL-8, ICAM1, and other chemokines and adhesion molecules), co-stimulatory molecules (CD40, CD80, CD86, and the inducible T–cell co-stimulator ICOS)[68]. In addition, inhibition of NF-κB can attenuate experimental colitis, illustrating its proinflammatory effect[69]. Other studies have shown that NFκB maintains gut epithelial homeostasis by exerting mucosa protective effects. For example, failure to activate epithelial cell NF-κB *in vivo* results in a significant increase in radiation-induced epithelial cell apoptosis[70]. Indirectly blocking NFκB activation *via* targeted deletion in *MyD88* gene worsens DSS induced colitis in mice in gut epithelial cells, this will be discussed in further detail below[71].

NEGATIVE REGULATORS OF INNATE IMMUNE RESPONSE

TLR signaling in the gut is kept under control by inhibitors of TLR signaling. One such negative modulator is Toll-inhibitory protein (Tollip). Prolonged exposure to LPS (most commensal organisms contain LPS in their cell wall) leads to elevated Tollip expression which makes intestinal epithelial cells poorly responsive to TLR-dependent response to commensal microflora[72]. Other negative regulators of TLR signaling include SIGIRR (single immunoglobulin-IR-related molecule or Tir8) and peroxisome proliferator-activated receptor-γ (PPAR-γ)[73,74]. Consistent with the biological plausibility that reduced expression of these inhibitors lead to IBD, intestinal inflammation is enhanced in SIGIRR-deficient mice[73]. In addition, expression of PPAR-γ is decreased in patients with active UC (but not CD) and up-regulated by 5-aminosalicylic acid[75,76].

Negative regulation of the host innate immune responses to the indigenous microflora maintains gut homeostasis. Key players in the negative mucosal regulation include interleukin-10 (IL-10) and IL-2, as evidenced by mice with deficiencies in these factors develop spontaneous intestinal inflammation, but are protected from intestinal disease when raised in germ-free environments[77-79]. This indicates that at steady state, pathologic consequences of immune activation by commensal microflora are constitutively inhibited by IL-10 and -2 dependent mechanisms. Colitis can be prevented when *IL-10*$^{-/-}$ mice are crossed to MyoD88 deficient mice, demonstrating that IL-10 maintains intestinal immune homeostasis by negatively regulating MyD88-dependent, commensal-induced inflammation[71]. In contrast, colitis in IL-2 deficient mice is independent of MyD88 and TRIF pathway. This indicates that the commensal dependent colitis in IL-2 deficient mice is driven by either nonclassical TLR-dependent signaling (independent of MyD88 and TRIF) or through a non-TLR innate pathway.

The development of colitis in IL-10 and IL-2 deficient mice is mediated by a pathologic T-helper type 1 (Th1) immune response through instructive signals induced upon innate recognition of microbes[79,80]. The Th1 response in IL-10 deficient mice is dependent on MyD88-dependent IL-12 or -23 p40 signaling[71,81]. In contrast, the aberrant Th1 response in the absence of IL-2 does not go through a classical microbial induction of MYD88/TRIF pathway and is also independent of IL-12 or -23 p40 signaling[71]. IL-27, a new bioactive member of the IL-12 cytokine family composed of an IL-12 p40 related polypeptide (EBI3) and a unique p28 subunit, may be the candidate instructive cytokine to drive a MyD88 independent Th1 response[71,82]. Its expression is associated with commensal-dependent, Th1 polarized colitis in IL-2 deficient mice[71]. Interestingly, IL-27 expression also is correlated with *IL-10*$^{-/-}$ Th1 polarized colitis, suggesting that IL-27 is regulated by both the TLR/MyD88 dependent and independent pathway[71].

The differential expression of TLRs may also in part explain how host intestinal mucosa discriminates between commensal (most of which are gram-negative bacteria that contain LPS in their cell wall) and pathogenic bacteria. Oral tolerance is believed to be controlled by antigen-presenting cells in the Peyer's patches by stimulating the activity of regulatory T-cells which suppresses adaptive immune response[83]. These Peyer's patch DCs produce IL-10 in response to inflammatory stimulation such as LPS, which is a ligand for TLR4. Unlike other TLRs, TLR5 is expressed mainly on CD11C+LPMC and not on conventional DCs or macrophages[84]. Interestingly, TLR4

expression is low in CD11C+LPMC[84]. Low expression of TLR4 in CD11C+LPMCs may prevent inappropriate immune response to commensal bacteria whereas TLR4 expression in Peyer's patch DCs leads to IL10 production and immune tolerance to commensal bacteria. In contrast, expression of TLR5 on CD11C+LPMC may allow mucosal inflammation following exposure to pathogenic flagellated bacteria.

ADAPTIVE IMMUNITY

It is established that the recognition of commensal-derived antigens by the adaptive immune system or its stimulation by the innate immune system play a key role in the pathogenesis of IBD. In particular, studies in genetically engineered systems and in inducible and adoptive transfer models of chronic intestinal inflammation have shown a key role of effector T lymphocytes for the inflammatory process in the gut. Importantly, both T helper 1 (Th1) and Th2 T cells have been shown to cause chronic gut inflammation, with CD having a predominately Th1 cytokine profile and UC having a Th2 cytokine profile (Table 2).

Differentiation of naïve T-cells into various T-cell subsets is summarized in Figure 3. IL-12, composed of P40 and p35 subunits, induces the formation of IFN-γ producing Th1 cells. In contrast, IL-4 induces STAT6 activation, promoting the expression of GATA-3, which feed forward to induce IL-4 expression and Th2 cell differentiation. IL-23 (composed of p19 and p40) promotes the development of an IL-17 producing CD4+ helper T cell subset through mechanisms that are distinct from the Th1 (STAT1, STAT4, and T-bet) and Th2 (STAT6) pathways[85,86]. Tregs, an immune-modulating subset of CD4+ T-cells, can suppress the differentiation and function of Th1 and Th2 cells. Interestingly, in the presence of IL-6, Treg-derived TGF-b can induce the differentiation of Th17 cells[87]. These T-cell subsets and their effect in IBD are discussed below.

T-CELL RESPONSES IN CROHN'S DISEASE

Immunologically it is proposed that inflammation in CD is the product of an exaggerated Th1 response mounted by genetically susceptible host in reaction to components of commensal bacteria[88]. The Th1 cytokine profile, including IFN-α, IL-12 (composed of p35 and p40), and TNF-α, is elevated in patients with CD[89-91]. Polarization of naïve T-cells towards Th1 type differentiation occurs through the activation of signal transducer and activator of transcription-1 (STAT1) and its stimulation of transcription factor T-bet, a Th1 "master switch" that upregulates and stabilizes the expression of IL-12[92-94]. IL-12 can amplify Th1 response by upregulating IL-18 on T-cells. IL-18 then stimulates NF-κB and AP1, and in synergy with STAT4 (activated by IL-12), transactivates IFN-α expression. The proposed pathway of TH1 polarization is summarized in Figure 3.

TNF-α and its family member are important in the pathogenesis of CD. Increased expression of TNF-α is seen in the intestinal mucosa of patients with Crohn's disease and Antibodies to TNF-α such as Infliximab decrease Th1 response in parallel with clinical and endoscopic healing[90]. The expression of LIGHT, another member of the TNF family, is also up-regulated in intestinal biopsies from CD patients and stimulation of the LIGHT receptor (herpesvirus entry mediator) induced IFN-γ production in lamina propria T cells, while blocking LIGHT inhibited CD2-dependent induction of IFN-γ synthesis, indicating a role for LIGHT in the regulation of IFN-γ and may be a way in which T-T cell interactions propagate intestinal inflammation[95,96].

TL1A is a recently identified member of the TNF superfamily that interacts with the death domain-containing receptor DR3 and found to be a strong co-stimulator of T-cells. Activation of T-cells by IL-12/IL-18 induces expression of DR3[97]. In T-cells, ligation of DR3 by TL1A activates NF-κB and leads to powerful co-stimulation of IFN-γ[98]. Co-activation is most evident for CD4+/CCR9+ T-cells which are enriched in the lamina propria and intraepithelial lymphoid compartment of the small intestine[99,100]. These T-cells are also enriched in the peripheral circulation of patients with CD and Celiac disease. IL-12 and IL-18 can also augment IFN-γ production in CD4+/CCR9+ T-cells[101]. In addition, peripheral CD4+/CCR9+ T-cells have been shown to express surface membrane (sm) TL1A, which appears to co-stimulate IFN-γ production independent of, but in synergy with IL12 and IL 18 (Figure 3)[102]. A recent study has provided a genetic perspective on the role of TL1A in

Table 2 Cytokine profile in IBD

Innate Immune Response			Adaptive Immune Response		
Cytokine	Crohn's disease	Ulcerative colitis	Cytokine	Crohn's disease	Ulcerative colitis
IL-1β	I	I	IL-5	N	I
IL-6	I	I	IL-13	N	I
IL-8	I	I	IL-17	I	N
IL-12	I	N	IL-21	I	N
IL-18	I	I	IFN-γ	I	N
IL-23	I	N	TL1α	I	?
IL-27	I	N			
TNF-α	I	I			
Light	I	I			
TL1α	I	?			

IL: Interleukin; TNF: Tumor necrosis factor; IFN: Interferon; I: Increase; N: Normal.

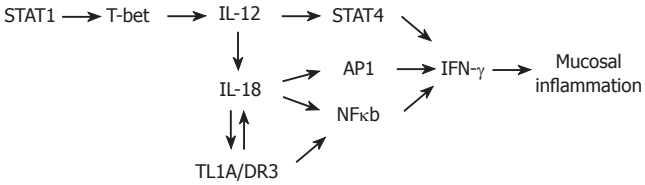


Figure 3 Th1 Polarization Pathway. Polarization of naïve T-cells towards Th1 cell subtype is initially induced by IL-12, a heterodimer of the p40 and p35 subunits. Activation of signal transducer and activator of transcription-1 (STAT1) and its stimulation of transcription factor T-bet, a TH1 "master switch" that upregulates and stabilizes the expression of IL-12. IL-12 can then amplify TH1 response by upregulating IL-18 on T-cells. IL-18 then stimulates NF-κB and AP1, and in synergy with STAT4 (activated by IL-12), transactivates IFN-γ expression.

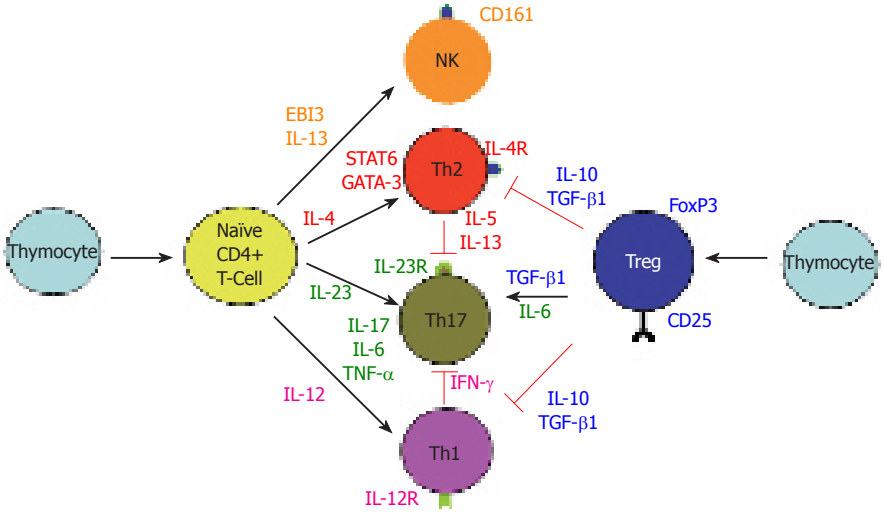


Figure 4 Differentiation of T-cell Subsets. Upon stimulation, naïve CD4+ T-cells differentiate into 3 main subsets, Th1, Th2, and Th17 cell. IL-12 induces the formation of IFN-γ producing Th1 cells. IL-23 promotes the development of an IL-17 producing CD4+ helper T cells. IL-4 induces STAT6 activation, promoting the expression of GATA-3, which feed forward to induce IL-4 expression and Th2 cell differentiation. An EBI3-associated cytokine was hypothesized to be necessary for activation of IL-13 producing NK T-cells. Tregs, an immune-modulating subset of CD4+ T-cells, can suppress the differentiation and function of Th1 and Th2 cells. Interestingly, in the presence of IL-6, Treg-derived TGF-β can induce the differentiation of Th17 cells.

the human TH1 mucosal response in CD. This genome-wide association revealed a highly significant association of SNP haplotypes of the TL1A gene with CD in a large cohort of Japanese patients as well as in two separate, smaller European cohorts[103]. These findings suggest that TNF mediated signaling is an important immune regulatory mechanism in mucosal inflammatory responses.

The IL-23/IL-17 immune axis

A subset of T cells producing IL-6 and IL-17, now known as Th17 cells, has emerged as an important mediator of the T-cell response in gut inflammation (Figure 4). IL-17 is a proinflammatory cytokine that enhances T-cell priming and stimulates fibroblasts, endothelial cells, macrophages, and epithelial cells to produce multiple pro-inflammatory mediators such as IL-1, IL-6, TNF, NOS2, metalloproteases, and chemokines[104]. IL-23 (composed of p19 and p40) promotes the development of an IL-17 producing CD4+ helper T cell subset through mechanisms that are distinct from the Th1 (STAT1, STAT4, and T-bet) and Th2 (STAT6) pathways[85,86,105]. Through this pathway, IL-23 regulates inflammatory processes in several mouse disease models[106-108]. In addition, bacterial colonization stimulates IL-23 expression by ileal dendritic cells[109] and the levels of both IL-23 and IL-17 are increased in Crohn's disease tissue[110,111].

CD is thought to be mediated by Th1 cells because of high levels of IL-12 and IFN-γ are detected and treatment with anti-IFN-γ mAb or anti-IL12 (p40) mAB suppresses disease development[112,113]. However, as IL-23 shares the p40 subunit with IL-12, the improved clinical disease may also be an effect of IL-23 inhibition. To determine the contribution of IL-23 in intestinal inflammation, mice with deficiency of IL-23 ($p19^{-/-}$) or IL-12 ($p35^{-/-}$) were used. A recent study showed that the development of colitis was suppressed by IL-23p19 deficiency but not IL-12p35 deficiency in $IL\text{-}10^{-/-}$ mice (model of T-cell mediated gut inflammation)[114]. Administration of IL-23 accelerated the onset of colitis in $Rag^{-/-}$ mice reconstituted with $IL\text{-}10^{-/-}$ CD4+ T-cells[114]. The suppression of inflammation in IL-23p19 deficient mice is not due to impaired Th1 (preserved IFN-γ expression) or Th2 (preserved IL-4 expression) but likely due to IL-17 pathway[114]. Notably, IL-23 (but not IL-12) augments IL-17 and -6 expressions by anti-CD3 mAB-stimulated memory CD4+ T-cells, distinguishing the ability of IL-12 to stimulate naïve CD4+ T-cells[114]. Antibodies that neutralize IL-6 and IL-17 ameliorated the severity of intestinal inflammation in $Rag^{-/-}$ mice reconstituted with $IL\text{-}10^{-/-}$ CD4+ T-cells[114]. These observations indicate that IL-23 promotes development and expansion of a pathogenic IL-6/IL-17 producing memory-activated T-cell population that can trigger the inflammatory cascade leading to intestinal inflammation.

The above findings do not exclude the role of exaggerated Th1 response in CD since IL-12/IFN-γ and IL-23/IL-17 may be parallel pathways involved in inflammatory response. Interestingly, IL-12/IFN-γ and IL-23/IL17 pathways are mutually exclusive, since IFN-γ suppress IL-17 and vice versa (Figure 4)[104]. It is of pathogenic importance to consider all of the major immune pathways that are responsible for the development of CD. For example, therapeutic targeting of only the newly discovered IL-23/IL-17 immune axis may actually exacerbate CD by accelerating the IL-2/IFN-γ Th1 pathway.

T-CELL RESPONSES IN ULCERATIVE COLITIS

In ulcerative colitis, it appears that the T-cell response is Th2 dominant (IL-4, IL-13) and mediated by specialized cells such as NK T-cells. By determining the cytokine profile of lamina propria mononuclear cells (LPMC) isolated from tissue recovered from colonic resection from UC and CD patients, it is found that LPMC from UC patients secreted high amounts of Th2 cytokines IL-13 and IL-5[115]. The IL-13 and -5 LPMC cells bear NK specific markers CD161 and recognizes CD1d, indicating that they are NK T-cells[115]. These NK T-cells are "nonclassical" because they do not express invariant NK T-cell receptors characteristic of most "classical" NK T-cells. They express noninvariant (diverse) TCRs that recognize antigens in association with CD1d, a MHC class I-like molecule present on the surface of dendritic cells

and on non-professional antigen presenting cells (APC) such as intestinal epithelial cells[116]. The "nonclassical" NK T-cells isolated from UC patients exhibited cytotoxicity towards an epithelial cell line (HT-29)[115]. This cell population possibly could be the cells causing epithelial cell cytotoxicity in UC described in 1960-1980s[117]. Together, these data show that ulcerative colitis is associated with an atypical Th-2 response mediated by a distinct subset of NK T-cells that produce IL-13 and are cytotoxic for epithelial cells. However, the extent to which this leads to the ultimate cascade of inflammation in ulcerative colitis remains to be determined.

Colonic epithelial cells express both CD1d and the Epstein-Barr virus-induced gene (EBI3), a protein related to IL-12p40. An EBI3-associated cytokine was hypothesized to be necessary for activation of IL-13 producing NK T-cells[118]. This is consistent with the fact that EBI3 is increased in mucosa from patients with UC as compared with CD or control tissues[119]. In addition, EBI3-deficient mice manifest poor Th2 responses and are resistant to the development of oxazolone colitis, a Th2 colitic murine model[120]. The EBI3-deficient mice have markedly reduced numbers of NK T-cells while the number of both naïve and mature CD4+ and CD8+ cells represerved[120]. These collective data indicate EBI3-associated cytokine (e.g. IL-27) may be necessary for the development of the Th2-cytokine-producing NK T-cells in both UC and oxazolone colitis.

CONCLUSION

Available evidence indicates that IBD is the result of dysregulated immunogenetic parameters that depends on impaired coordination between luminal microorganisms, gut epithelium, and the host immune system in genetically susceptible individual (Figure 1). These immunogenic parameters are complex and biologically divergent, which explains the heterogeneous clinical manifestations and lack of a universal therapeutic response to any single agent. The challenge for the future is to better understand the precise molecular mechanisms of disease immunopathogenesis. This can be achieved by identification of novel immunogenetic parameters, characterization of the heterogeneous parameters, and integrated multiparameter assessment of distinct aspects of the disease biology that are already known. These multi-disciplinary approaches are likely to stratify this complex disease into distinct subgroups that are more biologically homogenous with more defined pathogenic parameters and guidance for a more predictable response for individualized IBD therapy.

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TOPIC HIGHLIGHT

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Role of the intestinal barrier in inflammatory bowel disease

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Abstract

A critical function of the intestinal mucosa is to form a barrier that separates luminal contents from the interstitium. The single layer of intestinal epithelial cells (IECs) serves as a dynamic interface between the host and its environment. Cell polarity and structural properties of the epithelium is complex and is important in the development of epithelial barrier function. Epithelial cells associate with each other *via* a series of intercellular junctions. The apical most intercellular junctional complex referred to as the Apical Junction Complex (AJC) is important in not only cell-cell recognition, but also in the regulation of paracellular movement of fluid and solutes. Defects in the intestinal epithelial barrier function have been observed in a number of intestinal disorders such as inflammatory bowel disease (IBD). It is now becoming evident that an aberrant epithelial barrier function plays a central role in the pathophysiology of IBD. Thus, a better understanding of the intestinal epithelial barrier structure and function in healthy and disease states such as IBD will foster new ideas for the development of therapies for such chronic disorders.

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Key words: Intestine; Barrier function; Tight Junction; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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INTRODUCTION

The functioning of the gastrointestinal tract with its balanced microflora depends on the establishment and preservation of distinct compartments which are lined by a sheet of epithelial cells. The gastrointestinal epithelial lining consists of a monolayer of columnar cells that are held together by circumferential intercellular junctions to form a selectively permeable barrier to the luminal contents. Thus, the epithelium prevents unwanted solutes, microorganisms, and luminal antigens from entering the body[1,2]. Components of the intestinal mucosal barrier include luminal secretions such as mucus which is secreted on the apical surfaces of epithelial cells, followed by IECs with lipid plasma membranes, specific membrane transport systems responsible for transepithelial passage of different molecules, and the stromal compartment below the epithelial layer. The intestinal barrier has to be permeable for nutrients and macromolecules which are important for growth and development. At the same time, it has to provide an effective barrier to harmful macromolecules and microorganisms. The mechanisms that have evolved to deal with these physiological events are extremely complex. On the one hand, the structural and functional properties of the epithelium limit the amount of antigens reaching the surface of the epithelium. On the other hand, both specialized cells of the follicle-associated epithelium (M-cells) and dendritic cells sample luminal antigens that are then delivered to the cells of the mucosal immune system thereby guaranteeing permanent immunosurveillance. An intercellular junction referred to as the tight junction (TJ) is located at the apical end of the lateral intercellular space and is considered a key player that regulates paracellular movement of fluid and solutes[3]. Altered TJ structure and epithelial permeability has been observed in IBD[2]. Additionally, pathogens and bacterial toxins influence epithelial permeability by modulating TJ proteins[4-6]. Although the permeability defects could conceivably be due to the marked apoptosis that occurs during the inflammation processes, numerous studies have clearly shown that epithelial cell apoptosis alone does not account for the entire permeability deficits[7-10]. In addition, the importance of the intestinal microflora and more specifically its composition in physiological and pathophysiological processes in the human GI tract is becoming more evident. New discoveries relate to the beneficial effects of normal microflora and probiotics in preventing gastrointestinal infections[11-13]. Probiotics released by bacteria may functionally modulate the intestinal epithelial barrier of the host by different mechanisms, including the competition of whole organisms for contact

with the epithelial surface as well as stabilization of the cytoskeleton and barrier function and the induction of mucin expression.

Some of the most recently available data are discussed in this review. This field is changing rapidly and it is increasingly becoming accepted that immunogenetics play an important role in the predisposition, modulation and perpetuation of IBD. The role of the intestinal environment and the enteric flora in particular appears to be of greater significance than previously thought. This complex interaction of genetic, microbial and environmental factors culminates in a sustained activation of the mucosal immune system. This is facilitated by defects in the intestinal epithelial barrier and its mucosal immune system and results in active inflammation and tissue damage. We will concentrate on the role of the intestinal barrier, its regulation and modulation in IBD.

THE INTESTINAL BARRIER

Mucosal integrity and repair

The small and large intestine have potent mucosal defense and repair mechanisms. These mechanisms include a fast rate of cell renewal, a capable mucosal blood flow, a continuous adherent mucus layer and the presence of regulatory peptides that can stimulate repair mechanisms. The epithelial monolayer which lines the intestinal tract originates from multipotent stem cells present in the crypts[14]. Four major IECs are generated by these multipotent cells: (1) the absorptive enterocytes (reviewed in[15]), (2) the goblet cells responsible for the assembly of mucins[16] and trefoil peptides needed for epithelial growth and repair, (3) the enteroendocrine cells which export peptide hormones (reviewed in[17]) and (4) the paneth cells which secrete antimicrobial cryptidins or defensins, digestive enzymes and growth factors[18]. Injury to the epithelial lining resulting in mucosal erosion/ulceration can occur following exposure to pathogens and chemical therapeutic agents, decreased mucosal defense such as an abnormal mucus layer or reduced production of growth regulatory peptides. The GI tract epithelium has a remarkable capacity to rapidly reseal superficial erosions by migration of epithelial cells, a process referred to as restitution. Epithelial cell proliferation contributes to resealing of larger ulcers. Wound closure is influenced by a diverse array of peptides and growth factors released into the milieu of the regenerating epithelium. For example, the mucosal integrity peptide, TGFα (transforming growth factor α) directly acts on the enterocytes to stimulate proliferation and migration. TGFβ, and the pancreatic secretory trypsin inhibitor (PSTI) protect the overlying mucus layer from excessive digestion by luminal proteases and are involved in maintaining normal mucosal integrity[19]. Epidermal growth factor has been proposed as mucosal protector, playing an important role in luminal surveillance and rapid response to injury[20,21]. Mucosal epithelial cells and paneth cells produce a variety of antimicrobial peptides (defensins, cathelicidins, cryptidin related sequence peptides, chemokine CCL20) and bacteriolytic enzymes (lysozyme, group Ⅱ A phospholipase A2) that protect mucosal surfaces and crypts containing intestinal stem cells against invading microorganisms[21-23]. Trefoil peptides found in the goblet cells of the intestine are a family of three small proteins (TFF1, 2 and 3) which bind to the membrane-anchored glycoproteins of the filamentous brush border glycocalyx of the IECs. These peptides promote cell migration and interact with mucins such as MUC2, suggesting cooperation between the two in epithelial cell protection[24] and preservation of mucosal integrity[25]. Such peptides are rapidly up-regulated at sites of injury and inflammation. Recent experimental data has shown that the physiological role of TFF2, a member of the gastrointestinal trefoil factor family is associated with modulation of the immune system[25] and that the TFF2 rhythm is impaired in cohorts of individuals known to suffer from gastric symptoms, like *H pylori* infection and sleep deprivation[26].

KEY PLAYERS OF THE INTESTINAL BARRIER

Components

Intercellular junctions in epithelial cells play a vital role in regulating mucosal barrier properties. Specifically, the epithelial AJC consisting of the TJ and adherens junction (AJ) (Figure 1) is important in regulating cell-cell adhesion and paracellular movement of fluids and solutes. TJs are continuous, circumferential belt-like structures that form a permeability barrier at the apical end of the intercellular space. TJs regulate vectorial transport of water and electrolytes across the intestinal epithelium and prevent leakage of macromolecules from the gut lumen[27,28]. Additionally, TJs restrict the diffusion of lipids and proteins between the apical and basolateral plasma membranes (fence function) thereby preserving cellular polarity and, in combination with transcellular vectorial transport processes, generate distinct environments in the opposing compartments across the epithelium. Lastly, TJ proteins play an important role in the overall epithelial differentiation of cells, and their deregulation has been observed in epithelial cancers[29-31]. By freeze-fracture electron microscopy, TJs are viewed as a series of anastomosing intramembranous strands, the complexity of which correlates with barrier properties of the epithelium[32,33]. The TJ is a highly dynamic structure that regulates physiologic processes such as glucose absorption[3] and undergoes rapid regulatory changes in response to inflammation[34]. The AJs reside immediately subadjacent to TJs and play an important role in cell recognition and in mediating intercellular associations[35,36]. Both the TJ and AJ are intimately linked in their regulation and function and have therefore been collectively referred to as the AJC. Lastly, subadjacent to the AJC are spot-like intercellular junctions referred to as desmosomes (DMs). Although DMs create strong intercellular associations important in the integrity of stratified epithelia such as the epidermis, the function of DMs in the intestinal epithelium is poorly understood.

At a structural level, all the above intercellular junctions consist of transmembrane proteins that affiliate with the cytoskeleton *via* cytoplasmic plaque proteins. The association of AJC proteins with an underlying perijunctional filamentous actin (F-actin) ring plays an

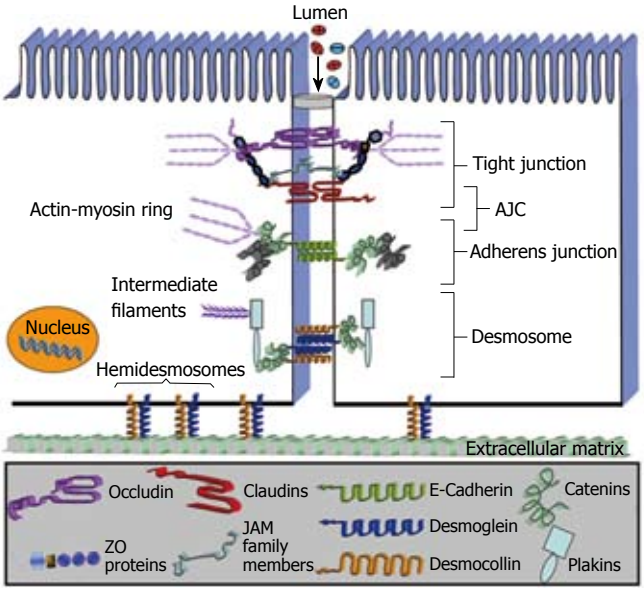


Figure 1 Intestinal epithelial intercellular junctions.

important structural and regulatory role in the AJC[37]. Key integral membrane proteins of the TJ include occludin, claudin family members, junction adhesion molecule-A (JAM-A) and the coxsackie adenovirus receptor (CAR). These proteins are believed to interact in a heterotypic or homotypic fashion in the extracellular space to create the selectively permeable epithelial barrier[37-39]. The intracytoplasmic domains of these TJ proteins interact with scaffolding proteins like the zonula occludens proteins (ZO), which contain several protein-protein interaction domains such as the PDZ domains (PSD 95, Discs large, ZO-1) important in mediating interactions of AJC-associated proteins. E-cadherin is a major transmembrane protein in AJs that associates with the apical perijunctional F-actin ring *via* cytoplasmic proteins in the catenin family[28,36,40]. The intestinal epithelial intercellular junctions are not static extracellular domains. Their adaptive mechanisms can undergo rapid regulatory changes in response to inflammation processes affiliated with IBD. Moreover the appropriate function of the AJC is coordinated by a complex array of signaling proteins which include the Rho and Rap family of GTPases, kinases and phosphatases[9,10,28].

INTESTINAL BARRIER AND REGULATION OF PERMEABILITY IN IBD

Most current treatments for IBD are aimed at reducing disease severity and at prolonging periods of disease-free remission by pharmaceutical suppression of inflammation. It appears that the pathophysiology of IBD is multifactorial. The enormous complexity of IBD pathophysiology therefore requires a systematic approach to identify the molecular events that cause and sustain the chronic, recurring inflammation. It has been suggested that the continuous stimulation of the mucosal immune system due to an increased permeability of the IECs may be the primary defect in patients suffering from IBD, whereas a healthy epithelium provides an effective barrier against luminal antigens. Increased paracellular permeability has been documented in the epithelial lining from both the acutely inflamed and chronically damaged areas of the intestine[41,42]. Animal studies support the tendency for development of inflammation in areas of the intestine lying beneath the permeability defect[43] or even for an increased permeability prior to the onset of a spontaneous intestinal inflammation[44]. It is well known that intestinal permeability is regulated directly through alteration of TJ proteins, or indirectly through effects on the cytoskeleton. A broad array of inflammatory cytokines have been reported to regulate TJs and barrier function by recruiting additional inflammatory cells into the intestinal wall and by re-distribution of TJ proteins in IECs and endothelial cells[45].

CROHN'S DISEASE

Besides genetic linkage like CARD15/NOD2 mutations on chromosome 16[46,47], OCTN1 and 2 mutations on chromosome 5 and DLG5 mutations on chromosome 10[48], abundant evidence indicates that increased intestinal permeability is implicated in the pathogenesis of CD[49]. The integrity of the intestinal barrier in patients with Crohn's disease is known to be compromised. Moreover, first degree relatives of patients with CD have been documented to have increased enteric permeability[50-52]. Interestingly, spouses of patients with CD suffered from increased intestinal permeability[53], which suggests that environmental factors may play a role. It has not been investigated whether these individuals develop CD or other gastrointestinal disorders. However, the debate continues if the increased enteric permeability is to blame for the development of CD or if the barrier defect is a consequence of an existing immune or inflammatory response. Animal models mimicking CD such as the SAMP1/Yit model, showed increased paracellular permeability across intestinal epithelial cells at an early stage of disease (three week old mice), prior to the onset of inflammation[54]. Transgenic animal models revealed the importance of E-cadherin in maintaining the epithelial barrier by demonstrating that dysfunction of AJ proteins contributed to an IBD-like process[55]. In addition, a number of studies have shown a potential role for inflammatory cytokines like TNF-κ and IFN-γ in directly increasing intestinal epithelial permeability. *In vitro* model systems have demonstrated that such pro-inflammatory cytokines influence barrier function by inducing disassembly of TJs in epithelial cells[7,9,10,56]. Furthermore, a role for TNF-κ in both apoptosis independent disruption of epithelial barrier function *via* alteration of TJs and upregulation of epithelial apoptosis (reversible by anti-TNF-κ antibody treatment) in the absence of changes in the expression of TJ proteins was reported, suggesting that TNF-κ may be one of the major links between the leaky bowel and Crohn's disease[57,58]. Soderholm *et al*[59] reported that non-inflamed ileac mucosa from patients with CD showed increased epithelial permeability and that increased endosomal uptake of antigens in ileac CD may be mediated by TNF-κ[60]. A recently published study

pertaining to the expression of TJ proteins (occludin and zonula occludens), alpha2-smooth muscle actin, TGF-κ with a cytoskeletal protein (F-actin) in the intestinal epithelium of patients with inflammatory bowel disease showed that latent dislocation of TJ proteins, without disturbance of the cytoskeleton in the inactive mucosa of patients with CD, may permit the invasion of gut antigens secondary to the functional disruption of TJs, that in turn could initiate an altered immune response[61]. Some reports have demonstrated that IFN-γ increases permeability across model intestinal epithelial cell lines[7,56]. Recently published data suggest that normally poorly invasive enteric bacteria may, in situations of inflammatory stress, exploit lipid raft-mediated transcytotic pathways to cross the intestinal epithelium because of INF-γ-induced disruption of TJs[62]. Although the mechanisms by which IFN-γ induces permeability changes across the epithelium are still incompletely understood, our data demonstrated that the permeability changes occur secondary to endocytosis of TJ transmembrane proteins occludin, JAM-A and claudin-1[9]. Furthermore, we believe that such endocytosis of TJ proteins is initiated by activation of the Rho GTPases and subsequent downstream activation of Rho kinase and actin-myosin II contraction[10].

ULCERATIVE COLITIS

UC is characterized by diffuse mucosal chronic inflammatory disease in the colon. Analogous chronic mucosal inflammation has been observed in animal models such as the mdr1a-/- mice. In this mdr1a-/- animal model a link between epithelial barrier dysfunction and development of colitis has been proposed[63]. Moreover, UC is associated with a mutation of the Toll-like receptor (TLR)-4 gene in humans, resulting in impaired lipopolysaccharide (LPS) signaling[64]. LPS comprises the major cell wall component of gram-negative bacteria and is mainly recognized *via* TLR-4. Stimulation of TLR-4 with bacterial LPS or other ligands[65] leads to activation of the NF-κB signaling system and subsequent induction of inflammatory responses[66]. Kiechl *et al*[67] showed that carriers of these mutations displayed an increased risk of gram-negative infections stressing that the predisposition of UC is associated with a genetic background. Besides these genetic factors, it has been demonstrated that an activated mucosal immune system leads to impaired epithelial barrier function and tissue destruction in patients suffering from UC[6,68]. In contrast to patients with CD, where the cytokines IFN-γ and TNF-κ play a central role in altering the epithelial barrier function, it still remains unclear which panel of cytokines regulates inflammation and induces epithelial barrier dysfunction in UC. Recently published data has shown that IL-13 produced by CD1-reactive natural killer T cells (NKT) played a central role in a murine model of colitis[69]. The IL-13 producing cells were also found in patients suffering from UC which suggests that this cytokine is one of the key mediators in the intestinal pathology of UC. Interestingly Heller *et al* showed that IL-13 mediates a drop of transepithelial resistance without any induction of necrosis in model IECs by increasing the paracellular permeability stressing the profound effect of IL-13 on epithelial barrier function[34]. Furthermore, it has been shown that IL-13 is produced in large amounts in the lamina propria of patients with UC. This is accompanied by increased expression of the pore-forming tight junction molecule claudin 2, which leads to the development of impaired barrier function for small cations and is thought to be responsible for the diarrhea in UC[70].

PROBIOTICS AND BARRIER FUNCTION IN UC AND ANIMAL MODELS OF COLITIS

Data from different *in vitro* and *in vivo* models support the involvement of luminal bacteria in mucosal inflammation and alteration of the intestinal barrier function especially in UC. In contrast to CD, ulcerative colitis is a disease involving mucosa only. Intestinal inflammation is accompanied by direct adherence of bacteria to this mucosal surface while the protective function of the mucus layer seems to be disrupted[71]. Studies have shown that bacteria like *E. coli*, *lactobacilli*, *bifidobacteria* and *streptococci* are able to interact with immunocompetent cells, using the mucosal interface and locally modulate the production of proinflammatory cytokines[72]. They may also directly change the structure of the intestinal epithelial barrier. Although the treatment of CD with probiotics has not demonstrated any sufficient results yet[73-75], the treatment of UC has shown encouraging data[76-81]. Generally the mechanisms by which probiotic microbial agents contribute to the protection of the inflamed intestinal epithelium involve two main categories: (1) competition for binding sites and inhibition of pathogen growth as well as epithelial attachment or invasion[82,83] and (2) stimulation of the mucosal immune system including the stimulation of anti-inflammatory cytokine levels and enhancement of the barrier function. Recent work provided evidence that protective effects of probiotic microorganisms in a DSS model of experimental colitis are mediated by DNA, which was recognized by the mucosal TLR9 receptor and this interaction consequently lead to an increased production of β-defensins. Defensins are known to be responsible for the destabilization and disruption of microorganism cell membranes, leading to an increase in permeability and leakage of small molecules[84,85]. To combat invading pathogens, phagocytes need to be recruited to sites of bacterial entry. Leukocyte recruitment occurs along gradients of chemotactic factors, including chemokines and defensin chemoattractants at nanomolar concentrations[86,87]. In addition, it has been shown that the probiotic compound VSL #3 was effective as primary therapy in a colitis model of IL-10 gene-deficient mice. A direct effect on epithelial barrier function was described. The treatment resulted in a normalization of colonic physiologic function and barrier integrity along with a reduction in mucosal levels of proinflammatory cytokines and a significant improvement in histological disease by secretion of soluble factors enhancing the barrier integrity[88]. This stabilization of the intestinal barrier function is an important target in the treatment of intestinal inflammatory disorders. Otte *et al*[89]

reported that the treatment of cultured IECs with VSL #3 lead to an increase of the transepithelial electrical resistance (TEER). In addition, this probiotic mixture was found to diminish Salmonella-induced alterations in the cellular cytoskeleton[90,91], including the distribution of the tight junction protein ZO-1. The stabilization of the cytoskeleton by regulation of tight junctions is important in the preservation of the epithelial architecture and, thereby, in the maintenance of the intestinal barrier, which might be affected by the MAPK pathway as postulated by the authors[89]. Interestingly, the incubation of IECs with these probiotics also induced the expression of several mucins, leading to less adhesion of microorganisms and its compounds like LPS to the epithelial surface. Together these housekeeping mechanisms might be responsible for the homeostasis of the intestinal barrier function during inflammatory disorders like UC.

CONCLUSION AND PERSPECTIVES

The pathophysiology of intestinal inflammation is multifactorial. Whatever the trigger, increased epithelial permeability plays a central role in the inflammatory process. The permeability changes and inflammation are intimately linked and play a central role in perpetuating the chronic mucosal damage observed in IBD. TJs do not merely represent static junctions between epithelial cells but are multifunctional protein complexes involved in numerous vital and diverse functions of epithelial cells. It is becoming evident that the TJs are extremely dynamic structures involved in developmental, physiological, and pathological conditions like CD and UC, where defects in mucosal integrity and repair remain key elements in initiation and perpetuation of the disease. Future studies will be focused on the mechanisms by which the epithelial barrier can be made "less leaky". The mucosal immune system is the central effector of intestinal inflammation and injury, with cytokines playing a central role in modulating inflammation and epithelial barrier function. Cytokines are, therefore, logical targets for IBD therapy. Finally, preliminary clinical trials of probiotic therapies in IBD may offer a valuable tool for prevention and control of IBD. Understanding the function and action of these probiotics will lead to the selection of useful probiotic strains for clinical application. As more work is directed at the function and modulation of the intestinal barrier, further potential therapeutic targets will provide more options to combat CD and UC.

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TOPIC HIGHLIGHT

Daniel C Baumgart, MD, PhD, *Series Editor*

Recent trends in the surgical management of inflammatory bowel disease

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Abstract

Surgery is required in the vast majority of patients with Crohn's disease (CD) and in approximately one-third of patients with ulcerative colitis (UC). Similar to medical treatments for IBD, significant advances have occurred in surgery. Advances in CD include an emphasis upon conservatism as exemplified by more limited resections, strictureplasties, and laparoscopic resections. The use of probiotics in selected patients has improved the outcome in patients with pouchitis following restorative proctocolectomy for UC. It is anticipated that ongoing discoveries in the molecular basis of IBD will in turn identify those patients who will best respond to surgery.

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Key words: Crohn's disease; Ulcerative colitis; Ileal pouch anal anastomosis; Laparoscopic colectomy

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INTRODUCTION

Significant strides have been made toward more effective medical management of inflammatory bowel disease (IBD) over the past several decades. Surgery, however, is still an essential component of the current multi-modality approach to both Crohn's disease (CD) and ulcerative colitis (UC). The current surgical approach to CD, with its emphasis on bowel conservation, was born out of decades of surgical experience. Early published series on the surgical management of ileal CD supported ileocolostomy with exclusion, the so-called "Berg" or "Mount Sinai" operation, as a safe and often therapeutic surgical approach. Concern that the remaining bypassed segment of diseased intestine placed the patient at risk for recurrent symptomatic CD and adenocarcinoma as well as an incidence of stump blow-out influenced a shift towards resectional techniques. Efforts to resect disease with margins free of microscopic disease gave way to a more conservative approach with the realization that radical resection did not result in lower rates of disease recurrence[1].The addition of strictureplasty techniques to the surgical armamentarium has furthered this trend towards surgical conservatism.

In contrast, the history of the surgical management of UC is marked by a shift from non-resectional strategies to the current emphasis on near-total resection of the colon and rectum. Early approaches to the surgical management of UC involved the construction of appendectomies to facilitate colonic irrigation or diverting ileostomies. The application of proctocolectomy with permanent ileostomy in UC addressed the morbidity associated with not resecting the diseased colon and became standard-of-care for most of the 20th century. The refinement of techniques for constructing a neo-rectum using the ileum in the 1980's led to the wide acceptance of total proctocolectomy with ileal pouch-anal anastomosis (IPAA) as a viable and often preferable surgical option.

Today, 70%-80% of patients with CD and 30%-40% of patients with UC ultimately require surgery. The current indications for surgery in both CD and UC are well described and will be reviewed in this article. In addition, we will focus on more recent developments in the surgical and peri-operative management of IBD including the increasingly broad application of laparoscopic techniques, multimodality therapy for anorectal CD, and the use of post-operative probiotics therapy after IPAA.

CROHN'S DISEASE

The surgical approach to CD is dictated, in large part, by the anatomic distribution of the disease. CD of the distal ileum with a varying degree of colonic involvement is present in 40% of patients. Isolated small bowel disease, isolated colonic, and isolated perineal and anorectal disease are present in 30%, 20%-25%, and 5%-10% of patients respectively. Gastroduodenal disease is not uncommon and has been endoscopically documented in 20% of

CD patients, usually with concomitant ileal disease. Symptoms related to gastroduodenal disease, however, are uncommon, and gastroduodenal disease necessitating operative intervention is rarer still[2].

Irrespective of its anatomic distribution, CD can be classified on the basis of histopathologic and pathophysiologic criteria. The three major subtypes of CD are fibrostenotic disease, which presents with varying degrees of intestinal obstruction, fistulizing disease, and aggressive inflammatory disease. The former two often require operative intervention, while the latter is primarily managed medically.

Operative intervention for CD is indicated when medical therapies fail to adequately alleviate symptoms, or when patients develop one of several complications of the disease; specifically, these include fistula, abscess, obstruction, and malnutrition. Persistent symptoms which compromise quality of life despite several months of aggressive medical management, or recurrent symptoms following repeated attempts to taper aggressive therapies are well described indications for surgery. Adverse reactions to medical therapies, most often steroids, sometimes prompt a more aggressive surgical approach to the control of CD symptoms as well.

Fistula formation is a common component of CD but is infrequently an isolated indication for surgery. Fistulas may be accompanied by abscesses, inflammatory masses, obstructive symptoms, and in rare cases peritonitis, all of which may prompt surgical intervention. Enterocutaneous fistulas and peri-anal or perineal fistulas may significantly compromise quality of life. Operative intervention to alleviate symptoms is often warranted even if these fistulas do not constitute a significant threat to a patient's physical well being. Enteroenteric fistulas are frequently discovered during surgical exploration for other indications. The presence of these fistulas does not mandate surgical repair unless they result in significant sequelae of malabsorption; namely malnutrition, dehydration or extremely frequent bowel movements. Enterovaginal and enterovesical fistulas may necessitate surgery if they are associated with significant symptomatology, vaginosis or urinary tract infections.

Obstruction is a relatively common indication for surgery. The specific character of obstructive symptoms is largely dependent upon the site of obstruction. Most commonly, terminal ileal narrowing causes chronic crampy abdominal pain associated with oral intake, often resulting in malnutrition. Additional less common indications for surgery in CD include acute hemorrhage, malignancy, and fulminant colitis[3].

SMALL BOWEL DISEASE

As stated previously, indications for operative intervention for CD of the small bowel include obstruction and fistula, particularly when accompanied by abscess or an inflammatory mass. When small bowel resections are undertaken, anastomoses should be constructed between segments of bowel grossly free of active disease. In the setting of multiple strictures, strictureplasty may allow for conservation of bowel length. Conventional strictureplasty involves the longitudinal incision of a stenotic segment of bowel with subsequent transverse closure of the enterotomy to increase lumen diameter. Resection and strictureplasty are often used in conjunction with the former technique applied to longer stenotic segments and the latter technique applied to shorter stenotic segments. Strictureplasty techniques aimed at addressing longer stenotic segments have been described in the recent surgical literature and are being applied with increasing frequency[4].

The operative management of small bowel fistulas is a complex subject; the surgical approach is dictated by the structures involved. Generally, fistulous tracts are transected, diseased intestine is resected, the contents of the tracts are evacuated, and necrotic tissue is debrided. In the setting of enterocutaneous fistulas, the opening at the skin is typically excised and allowed to close secondarily. When fistulas involve intra-abdominal organs free of Crohn's disease, the opening in the non-diseased organ is debrided and closed primarily[5].

COLONIC DISEASE

Complications of segmental CD of the colon may be addressed with segmental resection. Diffuse disease may require proctocolectomy with ileostomy, or total abdominal colectomy with ileorectostomy if the distal rectum is free of disease. In the setting of toxic colitis, total abdominal colectomy with construction of an ileostomy and Hartmann pouch is the preferred approach. In principle, the management of fistulous disease of the colon is similar to that described for fistulous disease of the small bowel. Colon with gross disease should be resected. Fistulous tracts to the colon from a diseased small bowel are typically managed with small bowel resection, excision and primary closure of the colonic opening.

ANORECTAL DISEASE

Typical manifestations of anorectal CD include fissures, fistulas, abscesses, and anal canal stenosis. Perianal disease resulting in fistulas and abscesses is frequently treated in stages. Perianal sepsis from abscess formation is addressed with incision and drainage. Subsequent abscess formation may be prevented by placement of seton drains. Fistulotomy and rectal advancement flaps are additional options in the management of chronic fistulas. Fistulotomy is specifically applicable to low fistulas. Rectal advancement flaps are less frequently successful in the CD population and candidates for these approaches must be carefully selected. The absence of significant rectal mucosal disease is a prerequisite for successful advancement flap coverage. The construction of a diverting ostomy may render active disease quiescent and is a useful option in selected patients with debilitating perianal or perineal disease who are poor candidates for a larger operation. Finally, proctectomy and proctocolectomy are appropriate options for patients with persistent, severe disease[6].

ULCERATIVE COLITIS

In contrast to CD, UC may be cured with surgery. The

specific indications for surgical intervention fall into two broad categories: emergent and elective. Emergent indications include massive hemorrhage, toxic colitis, toxic megacolon, and intestinal perforation. Elective indications include the inability of medical therapy to alleviate symptoms, severe malnutrition, the presence of dysplasia, and cancer.

EMERGENCY INDICATIONS

Toxic colitis, characterized by diffuse abdominal tenderness, tachycardia, fever, and leukocytosis is initially treated with fluid resuscitation, intravenous steroids and antibiotics. Surgery is indicated if clinical parameters do not improve with medical therapy in 48 to 72 h. The operation of choice in this setting is a subtotal colectomy and ileostomy with Hartmann pouch or rectal mucous fistula. Pelvic dissection is avoided to allow for a subsequent safe conversion to a proctocolectomy. Toxic megacolon is a variant of toxic colitis characterized by severe dilation of the colon. Toxic megacolon may be acutely managed with a subtotal colectomy and ileostomy. Construction of a skin-level transverse colostomy for decompression and a loop ileostomy for diversion has been described as an alternative approach in this setting. Colonic perforation is typically managed with a subtotal colectomy and ileostomy. Massive hemorrhage is similarly treated with a subtotal colectomy and ileostomy following proctoscopic confirmation that the majority of the bleeding is proximal to the rectum.

ELECTIVE INDICATIONS

Severe persistent symptoms that compromise quality of life are an indication for surgical resection as are complications of long term steroid dependence. Malnutrition and growth retardation are common indications for resection in the pediatric population. Finally, dysplasia or colorectal cancer detected by colonoscopy mandate surgical resection.

Surgical options for chronic ulcerative colitis include proctocolectomy with ileostomy, proctocolectomy with continent ileostomy, and proctocolectomy with IPAA. Proctocolectomy with ileostomy remains an important surgical option with curative potential in UC and relatively low morbidity when compared to proctocolectomy with IPAA. In healthy individuals who are motivated to maintain fecal continence and are willing to accept the potential associated morbidity, restorative proctocolectomy with IPAA is the procedure of choice. Though the necessity of a temporary diverting ileostomy has been called into question by some investigators, the majority of experienced surgeons routinely divert patients undergoing IPAA for a period of two to three months. The construction of continent ileostomies was more popular prior to the refinement of IPAA techniques. Continent ileostomies are prone to failure from valve slippage and this approach is only applicable in highly selected patients. Conversion to a continent ileostomy may be appropriate in patients with an IPAA who develop septic complications but are uncomfortable with the realities of a conventional ileostomy[7].

Table 1 Advances in the surgical management of IBD

Crohn's disease
Laparoscopic ileocecal resection
Multi-modality approach to anorectal CD
Ulcerative colitis
Laparoscopic restorative proctocolectomy
Probiotics

ADVANCES IN THE SURGICAL MANAGEMENT OF IBD

Crohn's disease

The role of laparoscopy in the surgical management of CD has been the focus of considerable investigation over the past decade. Several non-randomized trials suggested equivalent morbidity and mortality following laparoscopic or open ileocecal resection. Benefits of the laparoscopic approach, such as shorter length of hospital stay and lower rates of post-operative bowel obstruction, were suggested in some series. More definitive data in the form of two published prospective randomized trials have shed further light on these topics. In the study published by Milsom *et al*[8], 60 male patients were randomized to either laparoscopic or conventional ileocolic resection. Serial pulmonary function tests (PFT's) were measured post-operatively and were used as an objective surrogate for recovery. While the laparoscopic group's PFT's normalized more rapidly than did those of the conventional surgery group, the return of GI function and length of hospital stay was not significantly different between the two groups[8]. In the study reported by Maartense *et al*[9], sixty patients were randomized to either laparoscopic-assisted or open surgery. Importantly, post-operative care of the enrolled patients, regardless of which operation they received, was standardized. The primary outcome parameter was post-operative quality of life as measured by responses to two standardized questionnaires during a three month follow-up period. Secondary outcomes included operating time, morbidity, hospital stay, post-operative morphine requirement, and costs. Median operating time was longer (115 min *vs* 90 min, $P < 0.003$), median hospital stay was shorter (5 d *vs* 7 d, $P = 0.008$), and costs were lower in the laparoscopic group when compared to the open surgery group. Quality of life did not differ between the two groups[9]. In summary, there is no demonstrable difference in outcomes after open and laparoscopic ileocecal resection. Evidence is somewhat contradictory with regards to recovery time, but there is some evidence suggesting a more rapid recovery following laparoscopic ileocecal resection (Table 1).

As stated previously, 70%-80% of patients with CD eventually require surgery. Unfortunately, rates of disease recurrence remain high with a median time of ten years between a first and second bowel resection. A number of studies have proposed lower rates of disease recurrence after bowel resection when side-to-side stapled anastomoses are performed as opposed to hand-sewn end-to-end anastomoses. The widespread observation that disease recurrence invariably affects

bowel proximal to the prior anastomosis has led some surgeons to hypothesize that some element of relative obstruction at the anastomosis contributes to the pathogenesis of proximal disease. If this is the case, it follows that techniques allowing for a larger, more widely patent, anastomosis might lower recurrence rates. A number of retrospective, and non-randomized prospective studies suggest longer intervals of time prior to second resections when the stapled technique is employed[10]. A multicenter randomized trial is ongoing to investigate these observations.

Several interesting observations and advances have been reported regarding the operative treatment of anorectal CD. As mentioned previously, the placement of a draining seton is of considerable utility in the management of perianal fistulas. Perianal fistula recurrence following the removal of draining setons, however, is common. A combined medical and surgical approach to anorectal disease has been widely advocated. Specifically, the use of infliximab therapy in combination with examination under anesthesia and seton placement is supported by retrospective data published by Regueiro and Mardini[11], and by Topstad *et al*[12]. Irrespective of pharmacologic and surgical intervention, anorectal fistulizing disease remains a difficult problem as evidenced by the 44% rate of fistula recurrence after combined seton placement and infliximab therapy cited in Regueiro and Mardini's study. Surgical diversion is an often effective, if relatively radical, strategy for severe, medically refractory anorectal disease. Galandiuk *et al* reviewed their extensive experience in treating patients with anorectal CD. They identified the presence of anal canal stenosis and concomitant colonic CD as predictors of the need for permanent fecal diversion[13].

Ulcerative colitis

Laparoscopic surgery for UC has attracted considerable interest in recent years. Several case series and case-control studies established the feasibility of laparoscopic restorative proctocolectomy with ileal pouch anal anastomosis[14]. One randomized study comparing hand-assisted laparoscopic and open surgery for both UC and familial adenomatous polyposis has been published[15]. Maartense *et al* randomized sixty patients and documented their post-operative recovery at three months after surgery as measured by two standardized quality of life questionnaires. Secondary parameters included post-operative morphine requirement, operating time, morbidity, hospital stay and costs. Recovery in the two groups was equivalent. The laparoscopic operation took longer and was more costly[15]. At the current time, there is little evidence to suggest a benefit to laparoscopic restorative proctocolectomy as compared to the open operation with the exception of cosmesis. Interest in this approach, however, both in the medical and patient communities, remains great. With increased experience the role for this operation will, presumably, become more clearly defined.

Morbidity associated with restorative proctolcolectomy with IPAA remains significant. Pouchitis is the most common long-term complication following IPAA. This syndrome, most often characterized by increased stool frequency, urgency, and abdominal discomfort, remains poorly understood. The efficacy of antibiotic therapy in the majority of patients suggests an infectious etiology. Promising data supporting probiotic maintenance therapy for relapsing pouchitis after initial treatment with antibiotics was published by Gionchetti *et al*[16]. Forty patients were randomized to receive a probiotic called VSL#3 containing viable lyophilized bacteria including four strains of Lactobacillus, three species of Bifidobacterium and Thermophilus, or placebo. Over a nine month follow-up period, 15% of patients treated with VSL#3 compared to 100% of patients treated with placebo relapsed[16]. Similarly impressive results for VSL#3 were published by Mimura *et al*[17] VSL#3 has also been studied as prophylaxis against pouchitis during the first year following IPAA in a randomized prospective study. Of 20 patients randomized to VSL#3, 2 (10%) developed pouchitis within 12 months. Comparatively, 8 of 20 patients randomized to placebo (40%) developed pouchitis[18]. These results warrant further study of probiotics in the prevention of, and as maintenance therapy following initial therapy for, pouchitis.

CONCLUSION

Surgery remains an important component of the multi-modality treatment of IBD, required in 70%-80% of patients with CD and 30%-40% of patients with UC. Operative intervention for CD is indicated when symptoms are refractory to medical therapies, or when patients develop complications of the disease. Emergent indications for surgical intervention in UC include massive hemorrhage, toxic colitis, toxic megacolon, and intestinal perforation. Elective indications include refractoriness to medical therapy, severe malnutrition, the presence of dysplasia, and cancer. During the last decade significant efforts have been made to apply laparoscopic techniques to the surgical management of IBD. Additionally, significant advances in medical therapy have been made which promise to impact positively on outcomes following surgical interventions. The role of laparoscopy in the surgical management of CD has been the focus of considerable investigation. No significant difference in outcomes after open and laparoscopic ileocecal resection has been demonstrated but shortened recovery time following laparoscopic resection has been suggested in some studies. Except for improved cosmesis, there is little evidence to suggest a benefit to laparoscopic restorative proctocolectomy as compared to the open operation. Morbidity associated with restorative proctocolectomy with ileal pouch anal anastomosis (IPAA) remains significant. Pouchitis is the most common long-term complication following IPAA. Probiotic maintenance therapy for relapsing pouchitis after initial treatment with antibiotics and as prophylaxis against pouchitis during the first year following IPAA is promising and further investigations are warranted.

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TOPIC HIGHLIGHT

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Special issues in pediatric inflammatory bowel disease

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Abstract

The incidence of pediatric inflammatory bowel disease (IBD) is rising and recent advances in diagnostics and therapeutics have improved the care provided to these children. There are distinguishing features worth noting between early onset and adult onset IBD. Physical and psychosocial development remains a critical target for the comprehensive management of pediatric IBD. Children are not just little adults and consideration must be given to the stages of development and how these stages impact disease presentation and management. The final stage will be the transition from pediatric care to that of adult oriented care and special consideration must be given to make this a successful process. This review highlights special considerations in the management of the child with IBD.

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Key words: Inflammatory bowel disease; Crohn's disease

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INTRODUCTION

Inflammatory bowel disease (IBD) first presents in childhood and adolescence in approximately 20% of all cases. A European study reported that in the entire IBD population (children and adults) the incidence of Crohn's disease (CD) increased significantly (+23%), while the incidence of ulcerative colitis (UC) decreased (-17%)[1]. One of the best North American studies reported the incidence of IBD in Wisconsin children to be 7.05 per 100 000, with the incidence for Crohn's disease being 4.56, more than twice the rate of ulcerative colitis (2.14). An equal IBD incidence occurred among all ethnic groups, and children from sparsely and densely populated counties were equally affected and the majority (89%) of new IBD diagnoses were non-familial[2]. The median age of onset is 12 years and there appears to be a slight male predominance in the younger age group. Given the rise in incidence and the onset of disease coinciding with growth and development it is very important to highlight the considerations that should be taken into account when managing childhood onset IBD. This review with underscore the many unique facets involved in the presentation, diagnosis, treatment and psychosocial well being of the child with IBD.

ETIOPATHOGENESIS

The underlying pathogenesis of IBD in children appears to be similar to that in adult onset such that IBD results from a complex interaction of environmental, genetic, and immune factors. Genetics, however, may play an even greater role in disease onset and susceptibility in patients who present earlier in life. Despite research efforts, a gene specific to pediatric onset disease has not been identified. It does appear however that the NOD2 gene is similarly present in both 30%-35% of adult and pediatric CD patients. NOD2 was first identified in 2001. Although the true pathogenic role of NOD2 in CD remains unknown, it is an important gene involved in innate immunity which lends support to the notion that genetically determined defects in innate, and likely adaptive immunity alter the way our mucosal immune system interacts with our resident bacterial flora[3]. This dysregulated interaction leads to the adaptive immune response that is, in large part, responsible for the chronic inflammatory lesions, characteristic of patients with IBD. NOD2 has been well studied in the Caucasian population, however, the frequency of this gene among patients with CD of other racial/ethnic groups in the United States is not well known. Despite similar clinical characteristics significantly lower frequencies of read NOD2 mutations have been seen in African American and Hispanic children with CD compared with Caucasian children with CD[4]. There has been some evidence to suggest that the ethnic background of a child with CD may be associated with different genetic variants. A study from Israel suggested that G908R (SPN 12) allele-variant of the NOD2/CARD15 gene is closely related to the appearance of CD at a young age in Jewish Ashkenazi patients[5]. Research is ongoing to further examine the influence of

ethnicity on disease susceptibility and disease modification. Initial genotype-phenotype correlation studies in children demonstrated that NOD2 is associated with fibrostenosing CD and more rapid progression to surgery[6]. It appears that genetics is only part of the story when it comes to understanding the influences or risk factors at predicting the natural history of disease in pediatric patients. Initial work suggested that there are specific immune markers present in the serum of children with IBD[7]. These markers were initially used to help differentiate CD from UC and to aid in the distinction between functional GI symptoms and those due to underlying IBD, CD in particular. The concept that sero-reactivity to specific microbial antigens seen in a subgroup of patients with IBD likely represents a surrogate measure of an individual's adaptive immune response has resulted in this being an important area of investigation. Since reactivity to yeast oligomannan (anti-*Saccharomyces cerevisiae* antibody; ASCA) was first described in Crohn's disease (CD), the number of CD-specific antigens for which reactivity can be measured has expanded to include the *Pseudomonas fluorescens* related protein (I2), *Escherichia coli* outer membrane-porin (OmpC), and CBir1 flagellin (CBir1)[8-10].

The evolution of serum immune response from diagnostic markers to markers of disease behavior and perhaps prognostic information has resulted in studies that have shown that the presence and magnitude of immune responses in a given child is associated with more aggressive disease phenotypes and more rapid disease progression to complication and surgery. Larger scale studies are underway to confirm these preliminary findings and to further develop a risk assessment tool to identify children at risk of more aggressive disease and subsequent introduction of early intervention[11,12].

DIAGNOSIS

The diagnostic approach to IBD in children is a two-tiered approach. The first diagnostic question is whether the presenting symptoms are compatible with a diagnosis of IBD. In the face of classic, alarm, symptoms the differentiation between IBD and diseases that mimic the symptoms of IBD can be relatively simple. Diarrhea and abdominal pain are the most common symptoms in both CD and UC with rectal bleeding more commonly seen in UC and weight loss and anorexia more characteristic of CD. Perianal disease, like in adults, remains characteristic of CD. However unlike adults, growth failure is an important presenting sign in pediatric onset IBD, most commonly in CD patients. The standard diagnostic approach is employed including routine labs looking for signs of inflammation (ESR, CRP, platelet count) complete blood count with a focus on the hemoglobin for anemia and other labs such as iron panel and albumin to look at nutritional/absorptive state. Stools studies should be done to rule out infection especially in the presence of a travel history. Endoscopic evaluation and tissue diagnosis remains the gold standard[13]. It is recommended that all children undergo both an upper endoscopy and colonoscopy at the time of initial investigation. The findings on upper endoscopy, although often non-specific, may provide additional information in a patient with indeterminate disease of the colon especially if granulomas are found. A recent study found upper gastrointestinal inflammation in 29 of 54 children (22 CD; 7 UC)[14]. Epigastric and abdominal pain, nausea and vomiting, weight loss and pan-ileocolitis were predictive of upper gastrointestinal involvement. However, 9 children with upper gastrointestinal involvement were asymptomatic at presentation (31%). Overall upper gastrointestinal tract inflammation was most common in the stomach (67%), followed by the esophagus (54%) and duodenum (22%). Thus, absence of specific upper gastrointestinal symptoms do not preclude presence of upper gastrointestinal inflammation. It is important to biopsy macroscopically involved and non-involved tissue so to maximize the yield on the initial invasive evaluation. Small bowel radiography is also part of the initial diagnostic evaluation of pediatric IBD patients especially in CD patients. This is especially so for patients in whom ileal intubation was not successful at the time of the colonoscopy or diagnosis is indeterminate. It has been suggested that a normal small bowel radiography alone should not be used to rule out pediatric inflammatory bowel disease when the symptoms suggest it. Colonoscopy with terminal ileal intubation is feasible and safe; it should be attempted in all children with symptoms consistent with inflammatory bowel disease[15]. Radiographic evaluation of the small bowel has evolved to include MRI enterography and CT enterography. Recent advances in gadolinium-enhanced magnetic resonance imaging (G-MRI) have been developed to enhance the resolution of the intestinal mucosa and facilitate the differentiation of UC from CD[16]. Until the MRI scanners are faster and the radiologists are more comfortable interpreting the scans, MRI remains experimental and should be limited to use in centers that have a radiologists who are trained in this technology. CT enterography on the other hand is evolving into the best method of assessing the small bowel and large bowel. The use of Volumen for oral contrast allows for accurate visualization and distension of the mucosa. The radiation is somewhat greater than a standard CT scan however is less than a small bowel follow through with prolonged fluoroscopy[17]. Experience is growing among pediatric gastroenterologists in this area. Positron emission tomography (PET) using fluorine-18-fluoro-deoxyglucose to identify metabolically active tissues was evaluated as a simple noninvasive alternative to conventional studies in identification and localization of active intestinal inflammation in children with IBD[18]. The authors concluded that PET may not be able to replace conventional studies; however, it may be useful when conventional studies cannot be performed or fail to be completed. The cost associated with this test is an important limiting factor. White cell scans have also been proposed as a noninvasive way of evaluating active inflammation, however there can be many false negatives and advances in technology such as the video capsule endoscopy (VCE) provides a unique way of evaluating the small bowel mucosa with increased sensitivity and specificity[19]. VCE may be helpful in patients with persistent small bowel symptoms despite what has been reported as a normal small bowel x-ray particularly those

children with persistent growth failure. Moreover in cases of indeterminate colitis, children may benefit from more detailed small bowel visualization[20].

In the face of diagnostic uncertainty, such as in children presenting with symptoms compatible but not diagnostic of IBD, the pediatric approach tends to be less invasive then the approach to an adult patient presenting with similar symptoms. In this setting pediatricians tend to use non-invasive testing first to gather information that may increase the probability of disease and hence lead to more evidence to support invasive diagnostic testing. Other than routine laboratory tests as noted above, there are fecal markers, such as calprotectin and lactoferrin, and serological markers, such as ASCA and pANCA, that may aid in the differential diagnosis. Earlier reports demonstrated that fecal calprotectin correlated closely with the best invasive measures of colonic and small bowel inflammation in childhood inflammatory bowel disease and lends itself particularly to the monitoring and assessment of therapeutic interventions in children with inflammatory bowel disease[21]. Subsequent experience with calprotectin has yielded similar findings and in some practices is used frequently to differentiate functional from inflammatory GI disorders. Novel technologies have led to the development of a diagnostic algorithm to evaluate the sensitivity and specificity of serological immune markers as predictors of IBD *vs* non IBD. Historically these tests were accurate in differentiating IBD from non-IBD in 2/3 of cases[22]. The addition of novel immune responses and a change in technology may improve the diagnostic accuracy of non-invasive testing to close to 90%. Further validation studies in pediatric patients are needed to determine if indeed these tests will help pediatric gastroenterologist increase the likelihood of diagnosing IBD in the face of positive markers. In the adult cohort that these tests were validated in, it appears that disease was ruled out with approximately 90% certainty. This would be an important advance in pediatrics where invasive testing is avoided if possible.

The next tier in the diagnostic process of IBD is the differentiation between CD and UC. In the face of a classic CD presentation which includes small bowel localization, presence of granulomas, typical endoscopic and histological findings, the differentiation is simple. However there are some features of pediatric onset IBD that may pose more of a diagnostic dilemma when disease is confined to the colon. Despite the classic teachings of UC always involving the rectum, there are reports of rectal sparing in pediatric patients carrying a diagnosis of UC. The group from Boston reported that a significant proportion of children with new-onset UC had patchiness of microscopic features of chronicity (21% of patients), relative (23%), or absolute (3%), rectal sparing, and had little or no crypt architectural distortion in their rectal biopsies (8%). Of interest, these features were not observed in adult patients with UC. In addition, a higher proportion of children with UC initially presented with subtotal or with pancolitis compared with the adults[23]. Another study demonstrated that children < 10 years of age had significantly less crypt branching, plasma cells in the lamina propria, cryptitis, crypt abscesses, and epithelial injury than adults. Endoscopic rectal sparing was also seen in another study in 23% of children with newly diagnosed, untreated UC, and this feature did not correlate with presenting symptoms. However, the presence of rectal sparing may indicate more aggressive disease that is less responsive to medical treatment[24]. These studies must be interpreted with caution as these patients likely have what has become known as UC-like CD and although clinically present with UC symptoms, there are endoscopic and histological features more characteristic of CD. In these cases a more accurate evaluation of the small bowel with video capsule endoscopy may be warranted and perhaps serological immune responses will be helpful in differentiating between CD and UC. Studies have shown that pANCA is more commonly seen in patients with ulcerative colitis and ASCA and OmpC are prevalent in CD patients. It was then found that pANCA may be present in 25% of all CD patients, predominantly in patients with disease confined to the colon. A novel CD marker, anti-CBir1, has been shown to be present in approximately 50% of pANCA positive CD patients but rare (< 5%) of pANCA positive UC patients[25]. This marker may help differentiate CD from UC in indeterminate cases. This may be important as the efficacy of therapies may differ between the 2 pANCA subgroups. Further research is underway to delineate further the role of these markers in predicting response to therapy and post surgical prognosis[26].

TREATMENT

Modern approaches to IBD therapy calls upon the need for disease modifying targets with the goal of mucosal healing . It is hypothesized that mucosal healing should reduce disease related complications and in turn maximize the quality of life of IBD patients. This may be even more of an important issue when treating children with IBD given the long term consequences of early onset aggressive disease presentations. Currently there are few therapies approved for children with IBD, however, the concepts of targeting the full spectrum of the inflammatory cascade and the notion of mucosal healing can be applied. The main goals of targeting therapy in pediatric IBD are to: (1) Maximizing efficacy; (2) Maximize adherence; (3) Minimize toxicity; (4) Maximize quality of life; (5) Maintain physical and psychosocial growth; (6) Prevention of disease complications.

Like their adult counterparts, the current approach to the treatment of children with IBD is based on disease severity. In other words the traditional "step up" approach is applied in most cases. This approach also takes into account medication safety as the more milder/less toxic medications are often employed first letting patients declare themselves failures necessitating navigating up the pyramid to more aggressive anti-inflammatory agents. Pediatric gastroenterologists are limited in their ability to interpret whether this is the correct strategy given few studies have been done in children to support use of these medications especially the Mesalamine based therapies. Often we are forced to extrapolate information from adult study populations which may not be applicable to a child.

Given the potential growth and development implications of persistent inflammation and corticosteroid dependency, efforts are made to maximize both anti-inflammatory and steroid sparing strategies. Aside from the potential growth effects, the esthetic changes associated with corticosteroid use can be devastating to a child. Nutritional therapy is typically unique to pediatric patients however compliance can be an obstacle to administration. A recent prospective, 10-week open-label trial in children with active naive CD randomized children to orally polymeric formula alone or oral corticosteroids. In this small study, children with active and recently diagnosed CD, a short course of polymeric diet was more effective than corticosteroids in inducing healing of gut inflammatory lesions[27]. Further large scaled studies are needed to further evaluate the short and long term benefits of this treatment strategy. In the USA, enteral nutrition is used more so as supplemental nutrition in the face of malnutrition and growth failure.

Recently, there has been a lot of discussion surrounding the notion of turning the therapeutic pyramid upside down, aka "top-down" therapy. In other words in patients who are candidates for corticosteroids, more potent biological therapies, such anti-Tumor Necrosis Factorα (anti-TNFα) therapies, may be considered as alternatives[28]. However the strategy of short term corticosteroids with 6-MP as bridge therapy may be a safer and equally effective strategy in this patient population. Markowitz *et al* demonstrated that a significant proportion of children were off steroids and in remission 600 days after the combination therapy was initiated[29]. If however the desired outcome of a steroid free remission is not achieved in the expected time frame (4-6 months) of this combination then at that time the introduction of a biological therapy should be considered. The hesitation to go directly to a biologic stems from the fact that the thiopurines work well in children and the serious safety concerns. There are definitely safety concerns associated with thiopurines as well, however, TPMT screening and metabolite monitoring enable clinicians to identify at risk patients and dose adjust so to minimize toxicity and dose escalate safely in patients with sub-therapeutic levels and not responding to their current dose[30]. The REACH trial was the first of its kind in pediatrics to evaluate in a multi-center fashion the efficacy and safety of infliximab in 113 patients[31]. The study was not powered for efficacy but the results do support its use in children with the response rate at 10 wk was close to 90% and a remission rate at 54 wk of approximately 50% which includes children off of corticosteroids when receiving drug every 8 wk as opposed to every 12 wk. The safety may have been more favorable among patients receiving the every 12 wk infusions, however the efficacy benefit of every 8 wk may outweigh its safety risks. The data lead to the approval of infliximab for children with luminal Crohn's disease. Clinical trials are underway to evaluate the efficacy and safety of infliximab in children with ulcerative colitis as well as adalimumab for pediatric CD. Weighing the risks and benefits of each therapy must be considered and should be communicated with the child and the family. New safety information has emerged which has already started to alter the approach to patients receiving infliximab. There have been 18 confirmed cases of hepato-splenic T cell lymphoma (HSTCL) reported in patients receiving combination thiopurines and anti-TNFα therapy. Although rare, the majority of cases are fatal which has forced pediatricians to rethink their approach to this patient population. This calls into question of concomitant immunomodulation for the purpose of immunogenicity and perhaps improvement in response rates and how it relates to safety. In REACH all children had to be on concomitant therapy to be eligible so it is not known whether monotherapy would have been as effective. In the adult ACCENT1 trial only one-third of patients were on concomitant thiopurine or methotrexate and at the end of one year the remission rate off corticosteroids was only 24%[32]. Although the median duration of disease at the time of a patient's first infliximab exposure was much lower in REACH as compared to ACCENT 1 and the patient populations are different, one must question as to whether there is an efficacy advantage of the combination as opposed to monotherapy. A significant proportion of children are being removed from thiopurines and continuing the infliximab and some clinicians are extrapolating data from the rheumatoid arthritis literature that suggests that low dose methotrexate (7.5 mg po weekly) is associated with increased through infliximab levels and decreased levels of antibodies to infliximab[33]. There is no data among pediatric patients and further research is needed to validate this strategy. Methotrexate in any form of administration has not been common place in pediatrics. There was always the notion that injections are traumatizing to children and that the oral form may be associated with treatment limiting nausea and the bioavailability was inferior to that when given subcutaneously or intramuscularly. A more recent report however showed that the bioavailability of methotrexate in patients with inflammatory bowel disease is no different from that observed in other disease states[34]. Perhaps the key to deciding the best strategy for an individual patient will be in identifying the at risk patient up front whose risk of untreated progressive disease outweighs the risk of the medications. A recent study reported that imunomodulators are used in approximately 60% of children with CD within 1 year of diagnosis and suggested that lower serum albumin levels and hematocrit, and elevated erythrocyte sedimentation rate at diagnosis may predict the need for immunomodulators earlier in the disease course[34].

Future research will evaluate the role genetics and immune responses as immunological risk factors for aggressive disease. In other words, stratify patients into high risk and low risk which will in turn modify treatment approaches.

SPECIAL CONSIDERATIONS

Bones and growth

Consideration must be given to those issues which are unique to children. Most adult and pediatric gastroenterologists would agree that the presence of growth and pubertal delay is a key factor in the management of pediatric IBD. Puberty is the most dynamic phase of growth in childhood.

Maintaining adequate nutrition, minimizing inflammation and maximizing treatment off of corticosteroids remains an integral part of managing the potential growth stunting effects of active IBD, most specifically small bowel CD. Growth failure has been reported in up to 40% of children with CD and < 10% of UC. On occasion growth and pubertal delay is the only presenting sign of IBD and can precede any GI symptoms[36]. Growth failure is secondary to malnutrition with decreased intake contributing significantly to the malnutrition. Other factors such as increased GI losses, malabsorption, psychosocial factors and medication effects can certainly impact an individual's nutritional state. Ongoing inflammation with release of specific cytokines that suppress growth factors has been shown to be a very important determinate of growth failure. Evidence suggests that IL-6 mediates growth failure in children with Crohn's disease[37]. Nutritional supplementation and need for "catch-up" growth should be an important part of the evaluation of a pediatric IBD patient. A child's self esteem can be significantly impacted by growth failure and every effort should be made to make this a priority in the management plan. Administration of growth hormone was examined in a pilot study (7 patients) and did not demonstrate any effect on growth[38]. However the combination of growth hormone with nutritional supplementation in an IBD patients would likely yield positive results given the important of adequate caloric intake in patients with ongoing inflammation.

Defective bone mass accrual is another complication of chronic inflammatory disease and the major causative factor is growth failure. There are, however, other variables, such as physical activity, altered body composition, and disordered calcium and vitamin D metabolism which certainly play a role in bone mass. Persistent inflammation in the face of IBD can also impact the maintenance of bone mass. The method for assessment of bone mass in children is very important and what is currently used in adults does not apply to the pediatric age group. Bone mineral density (BMD) T-scores are appropriate for individuals that have reached skeletal maturity, but they should not be used in pediatric DEXA reports[39]. Instead, a Z-score should be calculated by subtracting the measured BMD from the expected BMD for individuals of the same age and sex, and dividing the result by the standard deviation. Additional adjustments may be needed in small or physically immature children (e.g., using height age or bone age instead of chronological age for Z-score calculation). It is important to note that a diagnosis of osteoporosis should not be made in children based on DEXA results alone. Experts in the field suggest that a Z-score of < -2.0 in children should be reported descriptively as "reduced bone mineral mass for age"[40]. Because of these limitations, total body bone mineral content may be clinically more useful than BMD in children, especially when studying patients over a longitudinal time frame. Quantitative computed tomography (QCT) is an alternative to measure BMD in both adults and children[41]. QCT offers the advantage of providing a true volumetric BMD, and it can distinguish the individual contributions of cortical and trabecular bone. QCT can be performed in conventional CT scanners, usually in the lumbar vertebrae. It involves a higher radiation dose than

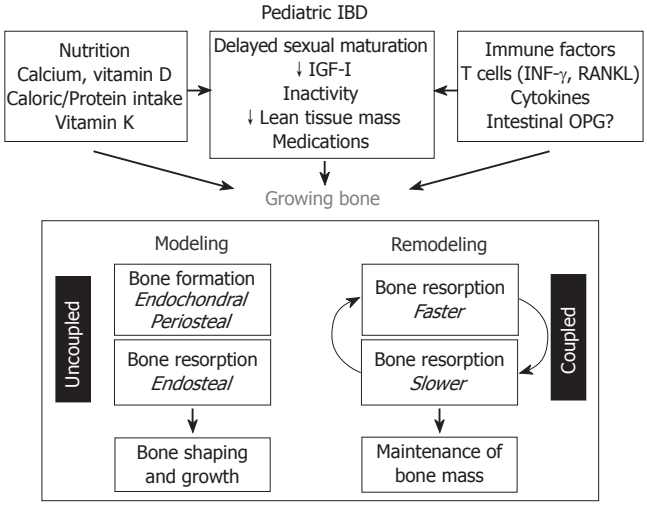


Figure 1 The effect of IBD on skeletal development.

DEXA. CT devices are available that measure BMD in the peripheral skeleton, with minimal radiation exposure, but the clinical significance of these measurements in children and adults with IBD is not known. Other experts in the field have shown that the apparent prevalence of osteopenia in children with IBD differs according to the method of data analysis used. Failure to account for bone age led to a label of moderate or severe osteopenia in 65% of cases. After adjustment for bone age, the proportion of children with osteopenia fell to 22%. Data suggests that children with IBD often have small bones for age because they have growth retardation. When DEXA data are interpreted with adjustment for bone size, most children were found to have adequate bone mass. Correct interpretation of DEXA is important for identifying children who may be at a real risk of osteoporosis[42] (Figure 1).

Quality of life and psychosocial issues

The burden of disease, both physical and psychosocial, imposed on children by Crohn's disease (CD) and ulcerative colitis (UC) can be considerable. In order to asses this burden the IMPACT questionnaire, a disease-specific measure of health related quality of life (HRQOL), was developed and validated for use in children and youth with IBD ages 10 to 18 years inclusive[43,44]. One study reported that the majority of patients perceived an improvement in HRQOL within 1 year of diagnosis and this improvement was in keeping with the overall improvement in disease severity[45]. Hester *et al* asked whether it may prove helpful to ask significant others or caregivers besides the patients themselves when evaluating health and the perception of health of patients[46]. This study reported that parents and children with IBD show high agreement when reporting observable aspects of the child's HRQOL. On the other hand, agreement was lower when it concerns more subjective aspects of HRQOL, such as social functioning and emotions. It is worth noting that children reported fewer problems than their parents. Through the CCFA, summer

camp programs are available for children with CD and UC. The goal of such camp programs is to allow children to participate in normal camp activities surrounded by their peers dealing with similar childhood issues. It has been suggested that camp may provide additional psychosocial benefits such as increased self esteem, altered perceptions and attitudes towards illness[47]. A prospective analysis of quality of life was completed at an overnight camp using the IMPACT Ⅱ questionnaire (Canada and United States) and the State-Trait Anxiety Inventory for Children. These questionnaires were administered to the campers at the beginning and at the end of a 1-wk camp to assess HRQOL and anxiety. No difference was found between anxiety scores before and after camp on either the state or trait anxiety inventories. HRQOL, however, improved after the week of summer camp suggesting that camp may normalize the chronic illness experience[48] (Figure 2).

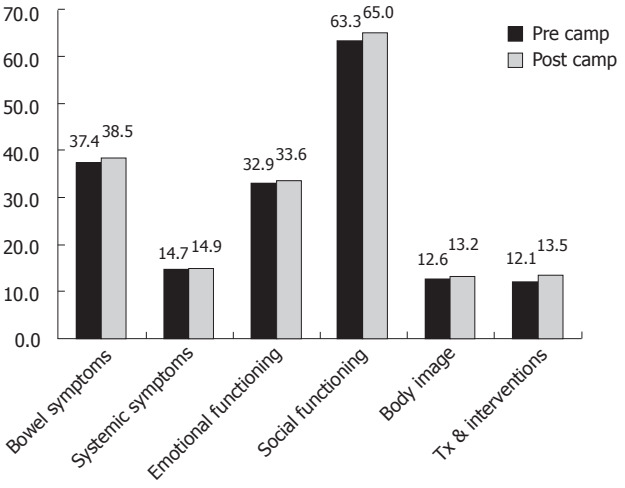


Figure 2 The impact of summer camp on HRQOL in children with IBD.

Psychosocial functioning and medication adherence

When comparing self-reported psychosocial functioning (behavioral/emotional functioning, social competence, self-esteem, stress coping strategies, and social support) of children with IBD to that of healthy children, it appears that most children with mild IBD report normal psychosocial functioning that is similar to that of healthy children[49]. Although rates range, adherence rates among chronic disease in children are typically reported to be approximately 50% with adherence being the lowest in adolescence and when maintenance medications are used even when the disease is in remission[50]. There are multiple factors that impact adherence in pediatric chronic illness which makes it potentially a more complex issue than in the adult population. Parents are often responsible for ensuring their children take their medication, so when evaluating adherence, both the patient and parent must be considered. One study examined the reports of adherence to oral medications, parent-child concordance in reports of adherence, and factors associated with poor adherence in adolescents with IBD. Mean parent- and child-reported adherence scores fell between the "most of the time" and "always" categories, although perfect adherence was low. Among IBD-specific medications < 50% of children and < 40% of parents reported being always adherent to all medications and parent-child concordance was high. Family dysfunction and poor child coping strategies were associated with worse adherence and there appeared to be a trend between more behavioral/emotional problems and lower adherence. Based on these results the authors concluded that adherence should be monitored in families that lack appropriate child discipline and in children who cope by simply wishing stressors would go away[51].

Transition of care

The transition from pediatric to adult medical care of patients with IBD can be difficult for the child and care givers. Clinicians must be sensitive to this transition and the barriers it may present. It has been recommended that the child be approached from the commonly accepted developmental stages, roughly defined chronology by ages 11-13, 14-16, 17-19, and 20-23[52]. These are certain traits needed to make a successful transition of care and allow for the ability to navigate through the adult healthcare milieu. Communication is critical in order for the child and caregiver to anticipate the new roles each member will play in this transition. The process of transition should be a gradual and if the process is delayed the transition may be less successful as the time to prepare and anticipate change has been limited. It is not unusual for the process to be more difficult on the family/primary caregivers than the child themselves. Family members need to relinquish responsibility and the weaning process should begin fairly early in adolescence so that when the time comes to meet with an adult gastroenterologist the patient and caregiver are prepared. In preparation patients may want to put a portfolio together whereby key documents can be brought to their visit to minimize duplication of history taking and perhaps procedures and tests. Examples would include, medical summaries, procedure reports, surgical reports, medication history, recent laboratory results and health insurance information.

Immunization status

Immunosuppressants/immunomodulators have become an integral part of the medical management of IBD both in adults and children. Guidelines have been proposed that take into account the interplay of host susceptibility to infection and the safety and efficacy of immunization for vaccine-preventable diseases. The guidelines suggest that with the exception of live agent vaccines [MMR (measles, mumps, rubella) and Varicella (chicken pox)], most immunizations (killed/attenuated) can be safely administered to patients with IBD even when immune compromised. Moreover, protection against vaccine-preventable illness may be of even greater benefit to those at risk for morbid or lethal complications of infections because of an immune compromised state. It was concluded that for most patients with IBD, recommendations for immunization do not deviate from recommended schedules for the general population[53]. Highlights of the recommendations from this report are the following: (1) Standard recommended immunization schedules for

children and adults should be generally adhered to. (2) At diagnosis, children and adults should have complete review of immunization history for completeness. All patients with incomplete series should commence catch-up vaccination. (3) Children who are not immune by vaccination or acquired immunity through infection should receive varicella vaccine. (4) Live bacterial or viral vaccines should be avoided in immune compromised children with IBD: (a) Treatment with glucocorticoids (prednisone 20 mg/d equivalent, or 2 mg/kg per day if less than 10 kg, for 2 wk or more, and within 3 mo of stopping); (b) Treatment with effective doses of 6-mercaptopurine/azathioprine (effect on safety not established) and within 3 months of stopping; (c) Treatment with methotrexate (effect on safety not established) and within 3 months of stopping; (d) Treatment with infliximab (effect on safety not established) and within 3 months of stopping; (e) Significant protein-calorie malnutrition. (5) Whenever possible, adequate immune response (ie. Tetanus toxoid antibody, H influenza B antibody) should be ascertained for individuals who have required immunization while immune-suppressed. Repeat dosing may be considered when immune response to immunization is insufficient.

CONCLUSION

Approximately one-quarter of IBD presents in childhood, most commonly among the adolescent age group which corresponds to the most dynamic phase of growth. The disease can impact both physical and psychosocial development. Growth failure and bone health are important consideration in the pediatric age group. The diagnosis and treatment requires a comprehensive approach to improve the health related quality of life of children with IBD. Treatments are improving but individualized therapeutic strategies will be a critical part in the management of pediatric IBD so to maximize the therapeutic efficacy and minimize toxicity. Parents and patients must be prepared for the transition from pediatric to adult care and tools are available to improve the success of this process.

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Correlation of integrin β3 mRNA and vascular endothelial growth factor protein expression profiles with the clinicopathological features and prognosis of gastric carcinoma

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Abstract

AIM: To investigate integrin β3 mRNA and vascular endothelial growth factor (VEGF) protein expression in gastric carcinoma, and its correlation with microvascular density, growth-pattern, invasion, metastasis and prognosis.

METHODS: *In situ* hybridization (ISH) of integrin β3 mRNA and immunohistochemistry of VEGF and CD34 protein were performed on samples from 118 patients with gastric cancer.

RESULTS: The positive rate of integrin β3 mRNA in non-tumor gastric mucosa (20%) was significantly lower than that of the gastric cancer tissue (52.5%, χ^2 = 10.20, P < 0.01). In patients of infiltrating type, stage T_3-T_4, vessel invasion, lymphatic metastasis, hepatic or peritoneal metastasis, the positive expression rates of integrin β3 mRNA were significantly higher than those in patients of expanding type (P < 0.01), stage T_1-T_2 (P < 0.01), non-vessel invasion (P < 0.01), without lymphatic metastasis (P < 0.01), without hepatic and peritoneal metastasis (P < 0.01), respectively. In patients of infiltrating type, stage T_3-T_4, vessel invasion, lymphatic metastasis, hepatic or peritoneal metastasis, the positive expression rates of VEGF protein were significantly higher than those in patients of expanding type (P < 0.01), stage T_1-T_2 (P < 0.01), non-vessel invasion (P < 0.01), without lymphatic metastasis (P < 0.01), without hepatic and peritoneal metastasis (P < 0.01), respectively. It was found that the positive expression rate of integrin β3 mRNA was positively related to that of VEGF protein (P < 0.01) and MVD (P < 0.05), meanwhile the positive expression rate of VEGF protein was positively related to MVD (P < 0.05). The mean survival period in patients with positive expression of integrin β3 mRNA and VEGF, and MVD ⩾ 54.9/mm^2 was significantly shorter than that in patients with negative expression of integrin β3 mRNA (P < 0.05) and VEGF (P < 0.01), and MVD < 54.9/mm^2 (P < 0.01). Five-year survival rate in patients with positive expression of integrin β3 mRNA and VEGF, and MVD ⩾ 54.9/mm^2 was significantly lower than those with negative expression of integrin β3 mRNA (P < 0.05), VEGF (P < 0.05), and MVD < 54.9/mm^2 (P < 0.01).

CONCLUSION: Integrin β3 and VEGF expression can synergistically enhance tumor angiogenesis, and may play a crucial role in invasion and metastasis of gastric carcinoma. Therefore, they may be prognostic biomarkers and novel molecular therapeutic targets.

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Key words: Stomach neoplasms; Integrin β3; Vascular endothelial growth factor; Metastasis; Prognosis

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INTRODUCTION

Gastric cancer is the leading cause of death in China.

For patients with gastric cancer, metastasis is the most common cause of death and is a major obstacle to successful treatment[1]. The spread of tumor cells from a primary to metastatic site is a complicated and multistage process, which may include cell proliferation and migration, degradation of basement membrane, invasion, and adhesion[2]. Current clinical methods cannot accurately predict which patients will develop metastasis. To develop effective new strategies for the prediction, diagnosis and treatment of metastasis of gastric cancer, molecular mechanisms controlling metastasis must be identified.

Angiogenesis, the process leading to the formation of new blood vessels, plays a central role in cancer cell survival, local tumor growth, and development of distant metastasis. The degree of intratumoral microvascular density (MVD) is thought to influence tumor metastasis and consequently prognosis in various human cancers, including gastric cancer[3-6].

Recent investigations have shown that high expression of integrin β3 is positively correlated with invasion and metastasis of cancer cells and tumor angiogenesis[7,8], but only a few studies have investigated the relationship between integrin β3 and prognosis of gastric cancer. In our study, we evaluated integrin β3 mRNA and vascular endothelial growth factor (VEGF) expression in gastric carcinoma and its relationship to pathological markers such as MVD, infiltration, metastasis and prognosis of gastric cancer.

MATERIALS AND METHODS

Patients and specimens

A total of 118 patients (79 male, 39 female; aged 38-80 years, median age 57.8 years) who underwent gastrectomy for gastric carcinoma at Zhejiang Provincial People's Hospital from October 1990 to November 1998 were included in this study. Five-year follow-up data were obtained, and the follow-up ended in November 2003. According to the WHO standard classification (2002), there were 39 tubular adenocarcinomas, 19 papillary adenocarcinomas, 37 poorly differentiated adenocarcinomas 12 mucinous adenocarcinomas, and 11 signet ring cell carcinomas. These patients were also classified into well- and moderately-differentiated (G1 + G2, 70 patients), and poorly-differentiated (G3 + G4, 48 patients) types, based on the predominant differentiation mode, and classified into the expanding (51 patients) and infiltrating (67 patients) type. According to the AJCC TNM staging system (6th ed, 2002), there were 21 stage T_1, 26 stage T_2, 45 stage T_3, and 26 stage T_4; 89 patients with and 29 without vessel invasion; 84 patients with lymphatic metastasis and 34 without; 55 patients with distant metastasis (35 with peritoneal dissemination, 20 with hepatic metastasis) and 63 without. A control study was carried out in 20 samples obtained from non-tumor gastric mucosa 5 cm away from the primary tumor without hyperplasia or atypical hyperplasia.

Reagents

Integrin β3 probe: oligonucleotide probe labeled with digoxigenin was obtained from Chinese Wuhan Boster Biotechnology (MK1602). The target gene mRNA sequence of human integrin β3 is: (1) 5'-GACACCTGTG AGAAGTGCCCCACCTGCCA-3'; (2) 5'-GGATG ACTGTGTCGTCAGATTCCAGTACTA-3'; and (3) 5'-GCTAAATTTGAGGAAAGGCGCGCCAGAGC-3'. Immunohistochemical reagents: first antibody and EnVision kit (DAKO Denmark), mouse monoclonal antibody to VEGF (1:100) and CD34 (1:120); VEGF clone: JH121; CD34 clone: QBEnd 10.

In situ hybridization

The samples in our study were from histological sections from paraffin blocks. *In situ* hybridization was performed according to the manufacturer's instructions. This experiment used RNase-free conditions and APES-treated slides, which were baked at 60℃ for 2-4 h before use. The samples were cut at 4-μm intervals, dewaxed with xylene, hydrated by a series of ethanol solutions (100%, 95%, 80% and 70%); inactivated with endogenous peroxidase using 3% hydrogen peroxide for 10 min at room temperature; washed three times with distilled water, each for 5 min; digested with Pepsin Reagent for 30 min at 37℃; rinsed three times in PBS for 5 min each, rinsed once in distilled water; fixed for 10 min at room temperature using 1% paraformaldehyde plus 1/1000 DEPC, washed three times with distilled water; then incubated in 50 μL pre-hybridization solution for 4 h at 40℃; and then superfluous liquid was absorbed. Each slide was incubated with 20 μL hybridization solution (concentration of the hybridization probe was 2 ng/μL) overnight at 40℃; then washed twice with 2 × SSC at 37℃ for 5 min each, once with 0.5 × SSC at 37℃ for 15 min, once with 0.2 × SSC at 37℃ for 15 min, and once with 0.2 × SSC at room temperature for 15 min. These sections were incubated in blocking solution for 30 min at 37℃ and superfluous liquid was absorbed. Following that, the sections were incubated in mouse biotinylated anti-digoxigenin for 60 min at 37℃, washed three times with PBS for 5 min each; incubated in SABC for 30 min at 37℃; washed three times with PBS for 5 min each; incubated in biotin/peroxidase for 30 min at 37℃; washed three times with PBS for 5 min each; stained with DAB for 3 min at room temperature, and washed thoroughly. The slides were counterstained with Harris hematoxylin, washed, dehydrated in ethanol, cleared in xylene, and enveloped with neutral gum. Negative controls included: (1) hybridization solution without probe; and (2) specimens pre-treated with RNase.

Immunohistochemistry

The EnVision two-step method was done according to the manufacturer's instructions. Tissue sections were cut at 4-μm intervals, dewaxed with xylene, hydrated by a series of ethanol solutions (100, 95, 80 and 70%). Method of high temperature and high pressure (CD34: 0.01 mol/L Sodium Citrate buffering Solution, pH 6.0; VEGF: 0.01 mol/L EDTA buffering solution, pH 9.0) were taken to repair the needed antigen. The sections were washed with distilled water, washed three times with PBS for 5 min each, inactivated endogenous peroxidase using 3% hydrogen peroxide for 10 min at room temperature; washed three times with PBS for 5 min each; incubated overnight with dilute first antibody at 4℃, and rinsed three times with

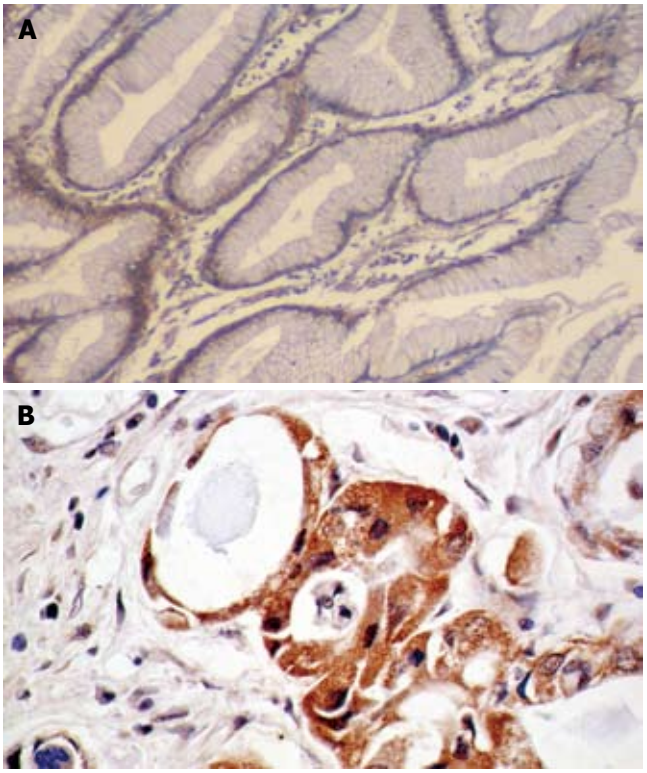


Figure 1 *In situ* hybridization of integrin β3 mRNA. **A**: Negative expression in the plasma of non-tumor gastric mucosa (ISH, × 100); **B**: Positive expression in the plasma of gastric adenocarcinoma (ISH, × 200).

PBS for 5 min each; incubated overnight with goat anti-mouse IgG antibody/HRP polymer for 40 min at 37℃, and washed three times with PBS for 5 min each. Visualization was achieved with DAB for 3 min. After counterstaining with Harris hematoxylin, the slides were dehydrated in 95% and 100% ethanol, cleared in xylene, and enveloped with neutral gum. The negative control included replacement of the first antibody with PBS, and the positive slides provided by the reagent kit were used as the positive control.

Results evaluation

Brown cytoplasm was indicative of positive expression of integrin β3 mRNA. The clearly-stained areas were chosen at high power (× 400), and > 200 cells were quantified in every visual field. According to the percentage of positive tumor cells, all these cells were scored as negative (-) (< 10% or no staining); weak positive (+) (11%-50%); positive (++) (51%-75%); or strongly positive (+++) (> 75%). Positive staining for VEGF was brown in the cytoplasm and/or cell membrane. These cells were scored as negative (-) (no staining); weak positive (+) (< 25%); positive (++) (26%-50%); strongly positive (+++) (> 50%), based on the percentage of positive VEGF cells. Assessment of MVD was performed as follows. Each slide was first evaluated at low power to capture the areas with the highest vascularization. Vessels with a clearly defined lumen or well-defined linear vessel shape, but not single endothelial cells were considered for microvascular assessment. We quantified positive vessels in five chosen fields (× 200) with the highest vascularization[3]. MVD was statistically recorded as the mean ± SD. The patients were divided into a high MVD ($\geqslant$ 54.9/mm^2) and low MVD (< 54.9/mm^2) group based on the mean MVD value (54.9/mm^2) of 118 gastric cancer patients.

Statistical analysis

Statistical analyses were performed using SPSS 10.0 software. Significant differences were compared with Student's *t* test. The χ^2 test was performed on the numerative data. Survival analysis was carried out using the Kaplan-Meier product-limit method, and survival curves were plotted. The differences were evaluated by the log rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Correlation between integrin β3 mRNA expression and clinicopathological features

The positive expression rate of integrin β3 mRNA in non-tumor mucosa was 20% (6/30). Positive signals were distributed in the cytoplasm of non-tumor glandular cell in gastric pit epithelium and the lamina propria, and the majority appeared weakly positive or positive. However, the positive expression rate of integrin β3 mRNA in gastric cancer was 52.5% (62/118). There were significant differences between the two groups ($\chi^2 = 10.20$, $P < 0.01$). Positive cancer cells were stained in the cytoplasm, and most tumor cells infiltrating the muscularis mucosa, serosal layers, extra-serosal layers, and greater omentum positively expressed integrin β3 (Figure 1). In patients of infiltrating type, poorly-differentiated cases, stage T_3-T_4, vessel invasion, lymphatic metastasis, hepatic and peritoneal metastasis, the positive expression rates of integrin β3 mRNA was significantly higher than those of the expanding type ($\chi^2 = 8.42$, $P < 0.01$), well-, moderately-differentiated case ($\chi^2 = 6.47$, $P < 0.01$), stage T_1-T_2 ($\chi^2 = 26.60$, $P < 0.01$), non-vessel invasion ($\chi^2 = 32.13$, $P < 0.01$), without lymphatic metastasis ($\chi^2 = 31.85$, $P < 0.01$), without hepatic and peritoneal metastasis ($\chi^2 = 20.48$, $P < 0.01$; $\chi^2 = 34.72$, $P < 0.01$) (Table 1).

Correlation between VEGF expression and clinicopathological features

VEGF was rarely expressed in normal gastric mucosa (Figure 2A). Of 118 gastric carcinoma patients, 64 positively expressed VEGF (54.2%), and VEGF staining was mainly located in the cytoplasm of tumor cells and usually observed at the tumor margins (Figure 2B). In patients of infiltrating type, stage T_3-T_4, vessel invasion, lymphatic metastasis, hepatic and peritoneal metastasis, the positive expression rates of VEGF protein were significantly higher than those of the expanding type ($\chi^2 = 10.44$, $P < 0.01$), stage T_1-T_2 ($\chi^2 = 34.19$, $P < 0.01$), non-vessel invasion ($\chi^2 = 39.96$, $P < 0.001$), without lymphatic metastasis ($\chi^2 = 34.71$, $P < 0.01$), without hepatic and peritoneal metastasis ($\chi^2 = 27.25$, $P < 0.01$; $\chi^2 = 52.77$, $P < 0.01$). However, no correlation was found between the different histological types (Table 1).

Correlation between MVD and clinicopathological features

CD34 staining was positive in vascular endothelial cells.

Table 1 Correlation of integrin β3 mRNA, VEGF protein and MDV with clinicopathological features

Clinicopathological parameters	*n*	Integrin β3 mRNA			VEGF				MVD		
		-	+ - + + +	χ^2	*P*	-	+ - + + +	χ^2	*P*	*n*/mm^2	*t*	*P*
Growth pattern	118			8.42	< 0.01			10.44	< 0.01		3.92	< 0.01
Expansive	51	32	19			32	19			49.45 ± 21.72		
Infiltrative	67	24	43			22	45			64.06 ± 18.76		
Histological grade (G)				6.47	< 0.05			3.49	0.062		1.25	> 0.05
G1 + G2	70	40	30			37	33			55.72 ± 21.56		
G3 + G4	48	16	32			17	31			60.70 ± 20.73		
Invasive depth				26.60	< 0.01			34.19	< 0.01		6.41	< 0.01
T_1-T_2	47	36	11			37	10			44.42 ± 19.96		
T_3-T_4	71	20	51			17	54			66.56 ± 17.23		
Vessel invasion				32.13	< 0.01			39.96	< 0.01		7.96	< 0.01
No	29	27	2			28	1			35.68 ± 14.04		
Yes	89	29	60			26	63			64.93 ± 18.07		
Lymph node metastasis				31.85	< 0.01			34.71	< 0.01		8.45	< 0.01
No	34	30	4			30	4			37.60 ± 15.73		
Yes	84	26	58			24	60			66.24 ± 17.24		
Distant metastasis									< 0.01			
No	63	48	15			50	13			42.81 ± 17.23		
Liver metastasis	20	4	16	20.48[a]	< 0.01[a]	3	17	27.25[a]	< 0.01[a]	73.40 ± 8.07	7.65[a]	< 0.01[a]
Peritoneal dissemination	35	4	31	34.72[b]	< 0.01[b]	1	34	52.77[b]	< 0.01[b]	75.79 ± 9.48	10.46[b]	< 0.01[b]

[a]Compared between patients with liver metastasis and those without distant metastasis. [b]Compared between patients with peritoneal dissemination and those without distant metastasis.

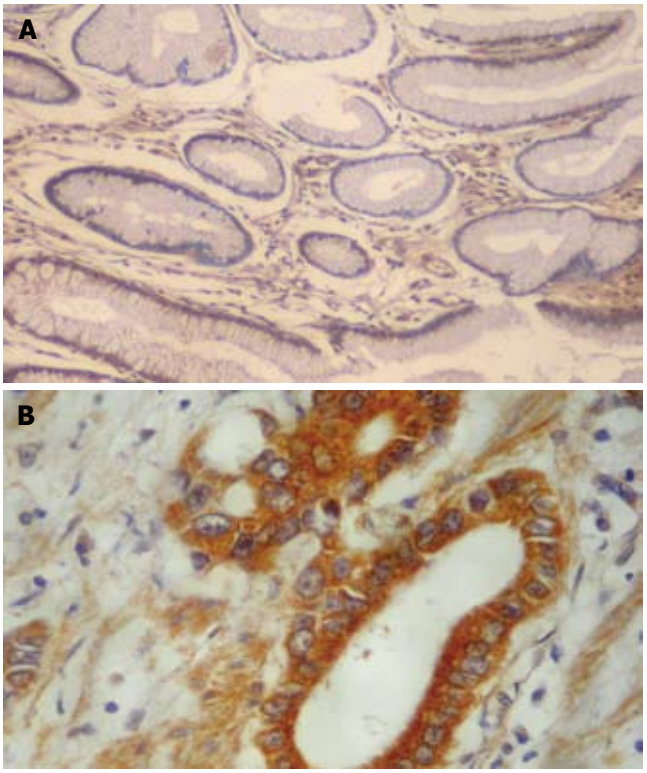


Figure 2 Immunohistochemical staining of VEGF. **A**: Negative staining in the cytoplasm of non-tumor gastric mucosa (EnVision, × 100); **B**: Positive staining in the cytoplasm of gastric adenocarcinoma with greater omentum infiltration (EnVision, × 200).

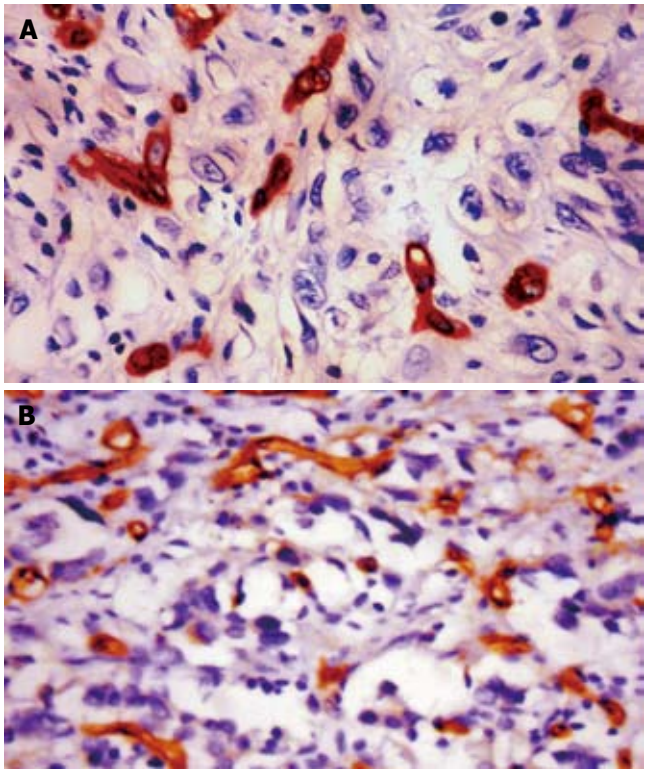


Figure 3 Microvessels in gastric cancer. **A**: Positive staining for CD34 in the vascular endothelial cells of gastric adenocarcinoma with MVD < 54.9/mm^2 (EnVision, × 400); **B**: Positive staining for CD34 in the vascular endothelial cells of gastric adenocarcinoma with MVD ≥ 54.9/mm^2 (EnVision, × 400).

Most microvessels in cancer tissue and surrounding carcinoma were brown or dark brown, strongly stained (Figure 3). In patients of infiltrating type, stage T_3-T_4, vessel invasion, lymphatic metastasis, hepatic and peritoneal metastasis, the mean MVD were significantly higher than those of the expanding type (t = 3.92, P < 0.001), stage T_1-T_2 (t = 6.41, P < 0.01), non-vessel invasion (t = 7.96, P < 0.01), without lymphatic metastasis (t = 8.45, P < 0.001), without hepatic and peritoneal metastasis (t = 7.65, P < 0.01; t = 10.46, P < 0.01), respectively. But no correlation was found within the different histological types (P > 0.05) (Table 1).

Table 2 Correlation of integrin β3 mRNA, VEGF protein and MVD with survival period

Groups		*n*	Mean survival period (mo)	*t*	*P*	5-yr survival rate (%)	χ^2	*P*
Integrin β3 mRNA	-	56	38.46 ± 29.20	2.27	< 0.05	71.4 (40/56)	5.60	< 0.05
	+ - +++	62	27.18 ± 24.73			11.3 (7/62)		
VEGF	-	54	41.70 ± 30.62	3.40	< 0.01	70.4 (16/54)	6.52	< 0.05
	+ - +++	64	24.80 ± 21.78			10.9 (7/64)		
MVD (n/mm^2)	< 54.90	47	44.70 ± 32.05	3.84	< 0.01	63.8 (17/47)	13.85	< 0.01
	⩾ 54.90	71	24.48 ± 20.40			8.5 (6/71)		

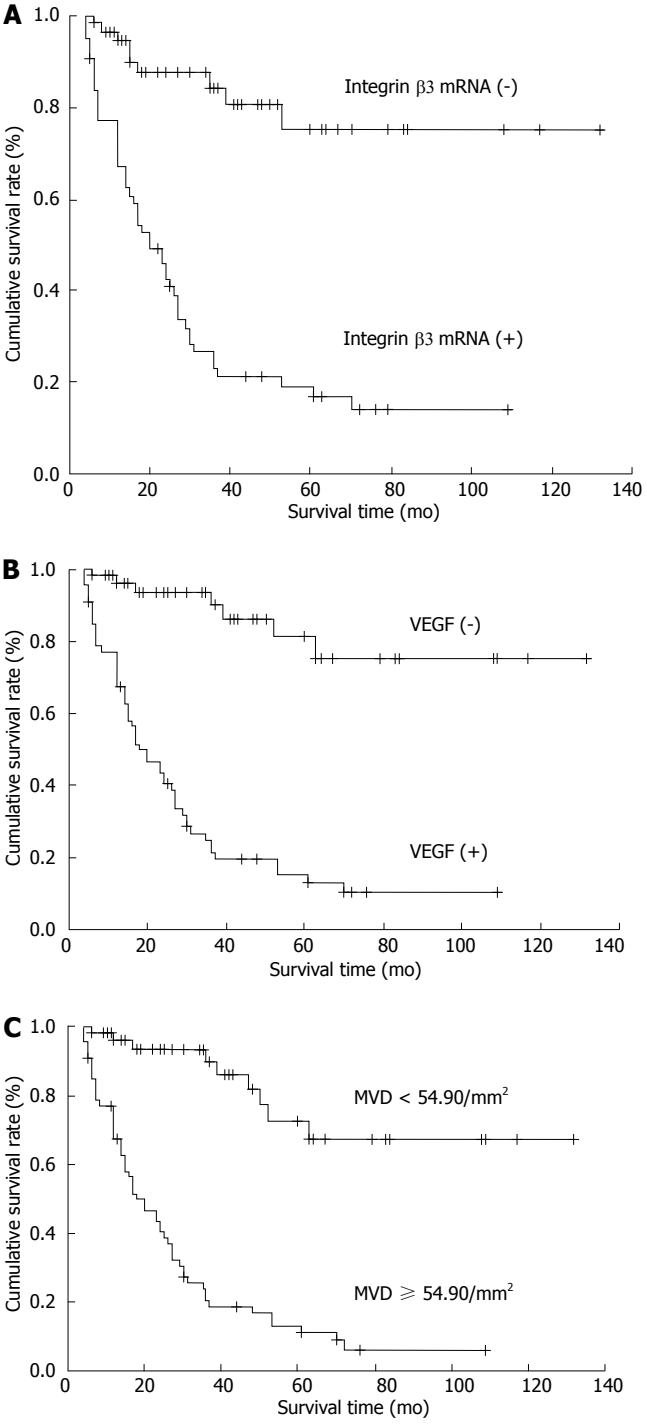


Figure 4 Kaplan-Meier survival curves. **A:** Survival curves with positive and negative integrin β3 mRNA expression in gastric adenocarcinoma ($P < 0.05$); **B**: Survival curves with positive and negative VEGF expression in gastric adenocarcinoma ($P < 0.05$); **C**: Survival curves with MVD ⩾ 54.90/mm^2 and < 54.90/mm^2 in gastric adenocarcinoma ($P < 0.01$).

Correlation between integrin β3 mRNA, VEGF protein expression and MVD

The mean MVD (70.45 ± 14.33/mm^2) in gastric carcinoma specimens with positive expression of integrin β3 mRNA was higher than that of the negative expression group (43.68 ± 18.77/mm^2; $t = 8.78$; $P < 0.01$). Similarly, the mean MVD in gastric carcinoma specimens with positive expression of VEGF (72.96 ± 1.38/mm^2) was higher than that of the negative expression group (37.76 ± 1.84/mm^2; $t = 8.78$; $P < 0.01$). In addition, the rate of VEGF in carcinoma tissues with positive expression of integrin β3 mRNA was 87.1% (54/62), which was significantly higher than that in the integrin β3 mRNA negative expression group (21.4%, 12/56; $\chi^2 = 51.48$; $P < 0.01$). Therefore, there was a positive expression relationship between integrin β3 mRNA, VEGF protein and MVD.

Correlation between integrin β3 mRNA, VEGF, MVD and prognosis

The survival time in patients with positive expression of integrin β3 mRNA, VEGF protein and MVD ⩾ 54.9/mm^2 was significantly shorter than that in patients negative for expression of integrin β3 mRNA ($P < 0.05$), VEGF protein ($P < 0.01$) and MVD < 54.9/mm^2 ($P < 0.01$). The five-year survival rate in patients with positive expression of integrin β3 mRNA, VEGF and MVD ⩾ 54.9/mm^2 was significantly lower than that of patients negative for expression of integrin β3 mRNA ($P < 0.05$), VEGF protein ($P < 0.05$) and MVD < 54.9/mm^2 ($P < 0.01$) (Figure 4A-C; Table 2).

DISCUSSION

Integrins are a major family of cell adhesion molecules, which mediate cell-cell adhesion or cell-extracellular matrix (ECM) adhesion, and affect signal transduction, cell proliferation, differentiation, survival and apoptosis[9]. Integrins are essential for invasion and metastasis of carcinoma cells. Integrins are transmembrane α β heterodimers that require divalent cations for their non-covalent association. To date, 19α and 8β subunits have been identified, and these can combine to form at least 25 different integrins. The ligands of integrins are type I and type Ⅳ collagen, laminin, fibronectin, vitronectin and fibrinogen. Integrins can bind to the ligands in ECM through the specific amino acid sequence in the ligands, which is a tripeptide that features in the integrin-interaction site of many ECM proteins, known as arginine-glycine-aspartic acid (RGD)[10,11]. Different adhesion

receptors play separate roles in intercellular homotypic or heterotypic adhesion, and the connection between cancer cells and ECM[12]. The connection between integrin αvβ3 and fibronectin enables membrane type-matrix metalloproteinase (MT-MMP) to be activated in human endothelial cells, and can also enforce cell invasion[13]. Ria *et al*[14] have found that integrin β3 interacts with fibronectin and vitronectin, which play a role in enhancing proliferation and migration of tumor cells.

Hosotani *et al*[15] have indicated integrin αvβ3 expression is elevated in pancreatic cancer, and expression in patients with lymphatic metastasis is significantly higher than that in patients without lymphatic metastasis. Furthermore, some studies have shown that, in malignant melanoma, ovarian, breast and prostate cancer, the invasive ability of tumor cells with positive integrin β3 expression is powerful, which suggests that integrin β3 is closely related to cancer invasion and metastasis[16-18].

Our study showed that integrin β3 mRNA expression in gastric carcinoma samples was stronger than that in non-tumor gastric mucosa, and the positive expression rate were significantly higher in the infiltrating type group, poor differentiation, perforating serosa layer, lymphoid node invasion, hepatic metastasis and peritoneal dissemination. This result was consistent with previous studies, and showed that increased expression of integrin β3 in gastric cancer influenced the adhesion between tumor cells and ECM. Moreover, it may influence signal transduction, thereby changing the biological behavior of tumor cells, and enhancing the potency of infiltration and migration[19].

Angiogenesis not only accelerates tumor growth, but also increases the opportunity of tumor cells for invading the vasculature, hence it promotes tumor metastasis. As the bridge between vascular endothelial cells and ECM, integrins play an important role in angiogenesis[8,20]. Previous studies have shown that integrin αvβ3 is minimally expressed on resting or normal blood vessels, but is significantly up-regulated in vascular cells within human tumors, and integrin αvβ3 has been implicated in tumor-induced angiogenesis[21,22].

CD34 protein, an endothelial-specific marker, was immunohistochemically stained, and MVD was determined in our study. Statistical analysis showed that β3 integrin mRNA expression ratio was positively correlated with MVD and VEGF. β3 integrin mRNA, VEGF and MVD were all associated with tumor clinicopathological features such as growth pattern, depth of invasion, vessel invasion, and lymph node and distant metastasis, which indicates that VEGF and β3 integrins contribute greatly to the progress of gastric cancer, by modulating angiogenesis. VEGF, the most specific and most potent regulator of angiogenesis, can stimulate the activation of endothelial cells, degradation of the matrix membrane, and migration and proliferation of cells to form new blood vessels[23]. Meanwhile, integrins and ECM are necessarily involved in proliferation and migration of endothelial and vascular smooth muscle cells[24-26]. Integrin β3 may enable VEGF to stimulate endothelial cell proliferation and capillary angiogenesis through activating VEGF receptor-2 (Flk-1) and VEGF receptor-3 (Flt-4)[27,28]. Therefore, integrin β3 and VEGF can enhance synergistically tumor angiogenesis, and lead to proliferation, infiltration and migration of tumor cells.

This study also demonstrated the relationship between integrin β3 mRNA, VEGF protein expression, MVD, survival period, and 5-year survival rate of gastric carcinoma patients. The results suggest positive integrin mRNA and VEGF protein expression, and MVD $\geqslant$ 54.9, are indicative of poor prognosis.

In summary, integrin β3 and VEGF synergistically enhance tumor angiogenesis, and may play crucial roles in invasion and metastasis of gastric carcinoma. Therefore, they can be used as biomarkers for diagnosis and prognosis, and as novel molecular therapeutic targets[29,30].

COMMENTS

Background

Metastasis is the most common cause of death and is a major obstacle to the successful treatment of gastric cancer. It is necessary to develop effective new strategies for the prediction, diagnosis and treatment of gastric cancer metastasis. Angiogenesis plays a central role in tumor growth and development.

Research frontiers

Recent investigations have shown that integrin β3 expression is elevated in pancreatic and breast cancer. High expression of integrin β3 is positively related to cancer cell invasion and metastasis and tumor angiogenesis, but few studies have investigated integrin β3 expression in gastric cancer, and the impact of integrin β3 and VEGF on prognosis.

Innovations and breakthroughs

Integrin β3 and VEGF expression can synergistically enhance tumor angiogenesis, and may play a crucial role in invasion and metastasis of gastric carcinoma.

Applications

This study cannot only define further the mechanism of metastasis of gastric cancer, but can also aid the development of tumor angiogenesis inhibitor. Integrin β3 and VEGF can be used as prognostic biomarkers and as novel molecular therapeutic targets.

Terminology

Angiogenesis is the process that leads to the formation of new blood vessels, and it plays a central role in cancer cell survival, local tumor growth, and development of distant metastasis.

Peer review

The authors studied integrin β3 mRNA and VEGF protein expression in gastric carcinoma by *in situ* hybridization and immunohistochemistry. They concluded that high expression of integrin β3 mRNA and VEGF protein were related to invasion and metastasis, and poor prognosis of gastric cancer.

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COLORECTAL CANCER

Inhibition of signal transducer and activator of transcription 3 expression by RNA interference suppresses invasion through inducing anoikis in human colon cancer cells

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Abstract

AIM: To investigate the roles and mechanism of signal transducer and activator of transcription 3 (STAT3) in invasion of human colon cancer cells by RNA interference.

METHODS: Small interfering RNA (siRNA) targeting Signal transducer and activator of transcription 3 (STAT3) was transfected into HT29 colon cancer cells. STAT3 protein level and DNA-binding activity of STAT3 was evaluated by western blotting and electrophoretic mobility shift assay (EMSA), respectively. We studied the anchorage-independent growth using colony formation in soft agar, and invasion using the boyden chamber model, anoikis using DNA fragmentation assay and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL), respectively. Western blot assay was used to observe the protein expression of Bcl-xL and survivin in colon cancer HT29 cells.

RESULTS: RNA interference (RNAi) mediated by siRNA leads to suppression of STAT3 expression in colon cancer cell lines. Suppression of STAT3 expression by siRNA could inhibit anchorage-independent growth, and invasion ability, and induces anoikis in the colon cancer cell line HT29. It has been shown that knockdown of STAT3 expression by siRNA results in a reduction in expression of Bcl-xL and survivin in HT29 cells.

CONCLUSION: These results suggest that STAT3 siRNA can inhibit the invasion ability of colon cancer cells through inducing anoikis, which antiapoptotic genes survivin and Bcl-xL contribute to regulation of anoikis. These studies indicate STAT3 siRNA could be a useful therapeutic tool for the treatment of colon cancer.

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Key words: Colon cancer; Invasion; Signal transducer and activator of transcription 3; Anoikis

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INTRODUCTION

Signal transducers and activator of transcription, STATs, are a family of transcription factors that transmit signals from cell surface receptors directly to the nucleus[1]. Activation of all the STAT proteins is caused by phosphorylation of a single tyrosine residue that leads to dimerization *via* an intermolecular SH2 phosphotyrosine interaction[2-5]. The dimerized STATs then translocate to the nucleus where they regulate gene expression by binding directly to high affinity DNA binding sites or by associating with other transcription factors[6-11]. They play a critical role in mediating cytokine and growth factor signaling involved in cell growth, differentiation and survival[12-14]. Among the seven members of the mammalian STAT family, STAT3 has been the most strongly implicated in oncogenesis[15].

Some studies showed that constitutively activated STAT3 is found in a wide variety of human tumors including multiple myelomas, breast, ovarian, prostate, and head and neck tumors[16-18]. Inhibition of STAT3 signaling with either dominant negative or antisense oligonucleotides against STAT3 suppresses the transformation process in some tumors[19]. Recent studies have shown that treatment of tumor cells with inhibitors of STAT signaling results in decreased cell viability and induction of apoptosis[20]. Together these findings demonstrate that STAT3 signaling

plays a critical role in both the transformation process and tumor progression in some types of cancer.

Recent reports showed that STAT3 has a significant association with tumor invasion and metastasis of a few cancers[21-25]. In renal cell carcinoma, the positive rate of the expression of p-STAT3 correlated well with the depth of tumor invasion and with metastasis[21]. In colorectal adenocarcinoma, p-STAT3 protein was significantly correlated with the depth of tumour invasion, venous invasion, lymph node metastasis, and increasing stages of the Dukes' classification[25]. In addition, blockade of activated STAT3 *via* ectopic expression of dominant-negative STAT3 significantly could suppress angiogenesis, tumor growth, and metastasis in pancreatic cancer[26]. Together these findings demonstrate that STAT3 activation might be a new potential target for therapy of human cancer metastasis. Recently it has been shown that STAT3 is constitutively activated in colon cancer[25], however, the role and mechanism of STAT3 signaling in metastasis of colon cancer remains elusive.

Colorectal cancer is the third most common malignant neoplasm worldwide[27] and the second leading cause of death due to cancer[28]. Despite recent advances in diagnostic and therapeutic measures, the prognosis of colorectal cancer patients with distant metastasis still remains poor. Liver metastasis is a major cause of morbidity and mortality in patients with colon cancer. Colorectal liver metastasis is associated with a very poor prognosis; most patients die within 2 years of diagnosis despite the availability of numerous therapies. To improve the choice of therapeutic strategy, it is critical that the mechanism of invasion and metastasis of colon cancer be clarified. Here we will show that knockdown of STAT3 expression by siRNA inhibit invasion ability and reduces anoikis resistance in colon cancer cells.

MATERIALS AND METHODS

Human colon cancer cell lines

The human colon cancer cell lines HT29, SW620 and SW480 were obtained from the Institute of Cell Biology, Shanghai, China. All cancer cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS). These studies were carried out with Medical Ethical Committee approval of Jiangsu University.

STAT3 siRNA and siRNA transfections

A double stranded siRNA oligonucleotide against STAT3 (5'-AACAUCUGCCUAGAUCGGCUAdTdT-3'; 3'-dTd TGUAGACGGAUCUAGCCGAU-5') was designed and synthesized by Dharmacon Research, Inc. Oligofectamine (Invitrogen, Inc.). In brief, 1 d prior to transfection, cancer cells were seeded, without antibiotics, into a 24 well plate, 1×10^5 cells/well, corresponding to a density of 60%-70% at the time of transfection. There were the following groups: siRNA groups: cells transfected with siRNA at different doses; control groups: cells treated with oligofectamine alone without siRNA. All transfections were performed in triplicate for each time point. At different times after the beginning of the transfection period, all cells were harvested and the following assays were performed.

Western blot analyses

HT29 cells were harvested and lysed in a buffer containing 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA (pH 8.0), 2 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 1 mmol/L Na_3VO_4. For western blot analyses, 30 μg of total extracted proteins were applied per lane before SDS-PAGE. Following transfer to nitrocellulose membranes, protein expression levels were detected using polyclonal anti-STAT3 (Santa Cruz, CA), anti- phospho-STAT3, anti-Bcl-xL and anti-survivin (Alpha Diagnostics International, TX). The expression of β-actin (Sigma-Aldrich, MO) was used as a normalization control for protein loading.

Electrophoretic mobility shift assay (EMSA) for STAT3-DNA binding activity

The STAT3-DNA binding activity was assessed by EMSA using the nuclear extract from the three cell lines. The sense strand that binds activated STAT3 protein was 5'-TCGACATTTCCCGTAAATC-3'. Double-stranded oligonucleotide was end-labeled with [γ-^{32P}] ATP using a T4 polynucleotide kinase according to the manufacturer's instruction. The final concentration of probe was 1.75 pmol/L. The labeled probes were then purified by G-25 spin columns. One microliter of ^{32}P-labeled STAT3 oligonucleotide was added to each reaction. For STAT3 specific tests, a 150-fold unlabeled STAT3 probe was applied as a competitor. The final volume of reaction was 20 μL, including 10 μg of nuclear extract and 5 × binding buffer. The reactions were placed on ice for 30 min. The 45 g/L nondenaturing acrylamide gel was pre-run in 1 × TBE buffer at 25 mA for 60 min. After loading of the samples, the gel was run at room temperature in 1 × TBE buffer at 25 mA for 90 min. The gel was dried on a gel dryer, then exposed to X-ray film overnight at -80℃ with intensifying screen. The protein-DNA complex was detected by autoradiography. The Quantity one software was used to analyze the scanned EMSA gel bands.

Anchorage-independent growth assay

For the anchorage-independent growth experiments, HT29 cells (8×10^3 cells/well) were seeded in 0.3% Difco Bactoagar (Difco, MI) supplemented with complete culture medium. This suspension was layered over 0.5 mL of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson, Italy). After 15 d, the colonies were stained with nitroblue tetrazolium, and colonies larger than 50 μm were acquired with a micro-Scopeman camera system (Moritex Europe Ltd, Italy) and analyzed with an Image-Pro Plus (Media Cybernetics, MD) computer program.

Cell invasion assay

Cell invasion assay was performed using Boyden chambers and 8 μm pore size polyvinylpyrrolidone-free polycarbonate filters coated with 25 μg/filter Matrigel (Beckton Dickinson). After transfection for 48 h, Sub-confluent HT29 control or HT29 STAT3 cell lines were harvested by a mild trypsinization, washed twice with Cellect medium and counted. Cells (2×10^5 viable cells/sample) were

allowed to invade Matrigel toward 10% FBS/Cellect at 37℃, 5% CO_2 for 3 h. At the end of the assay, cells on the lower surface of the filter were fixed in ethanol, stained with hematoxylin, and 10 random fields/filter were counted at 200 × magnification. Data represent the average of three experiments, all performed in triplicate.

Induction of anoikis

To prevent cell adhesion, 6-well plates were coated with a solution of polyhydroxyethylmethacrylate (poly-HEMA,Sigma-Aldrich), dissolved at 10 mg/mL in ethanol[29,30]. To coat 6-well plates, 3 mL of poly-HEMA solution was added to each well. Plates were kept at 37℃ for at least 3 d until the solvent had completely evaporated. To induce anoikis, 48 h after transfection, cells were harvested and moved to plates coated with poly-HEMA. 1×10^6 resuspended cells were cultured in DMEM medium containing 15% FCS for 12 h on poly-HEMA-coated dishes at 37℃ and 5% CO_2. Subsequently, cells were gently recovered and submitted to apoptosis detection assays.

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay

The TUNEL assay was performed according to a previous report[31]. Apoptosis index (AI) determination: apoptosis cells/total cell × 100%.

DNA fragmentation assay

For demonstration of internucleosomal DNA fragmentation, after cells were plated in 6-well plates coated with poly-HEMA for different times, the cells were harvested, washed with PBS solution at 4℃, and suspended in lysis buffer (10 mmol/L Tris-HCl pH 7.5, 10 mmol/L EDTA, and 0.2% Triton × 100). After incubation for 15 min at 4℃, samples were centrifuged at 13000 × *g* for 10 min at 4℃. The supernatant containing the fragmented DNA was precipitated with NaCl 0.5 mol/L and 1 volume of isopropanol for at least 1 h at -70℃. Samples were centrifuged at 13000 × *g* for 10 min at 4℃, and the pellet was washed once with 70% ethanol, and air-dried. The precipitates were dissolved in 10 μL TE-RNase (0.1 mg/mL) and incubated at 37℃ for 30 min. Finally, the samples were electrophoresed through a 1% agarose gel.

Statistical analysis

Statistical significance of results was evaluated by SSPS10.0 software. All results with P values $\leqslant 0.05$ were considered significant.

RESULTS

Effects of siRNA on the expression and DNA binding activity of STAT3 in the colon cancer cell line HT29

To inhibit STAT3 expression in colon cancer cells, we used the RNAi method adapted for mammalian cell culture by Elbashir *et al*[32]. A number of 21 bp double stranded RNAs to human STAT3 were synthesized and tested for their ability to knockdown STAT3 expression in colon cancer HT29 cells. The human colon cancer cell line HT29, harboring activated STAT3, was transfected with STAT3 siRNA using oligofectamine. Twenty-four and 72 h after transfection, the protein levels and DNA-binding activities of STAT3 were measured by western blot and EMSA, respectively. Transfection of cells with STAT3 siRNA (Figure 1A) resulted in a highly significant and reproducible decrease in STAT3 expression levels as judged by western blotting (Figure 1B). Figure 1A shows that STAT3 siRNA diminished STAT3 protein compared with control groups, accompanied by a significant decrease in STAT3 DNA-binding activity (Figure 1B). STAT3 knockdown by siRNA was found to be time dependent with the maximum effect achieved at 48-72 h of siRNA treatment (Data not shown).

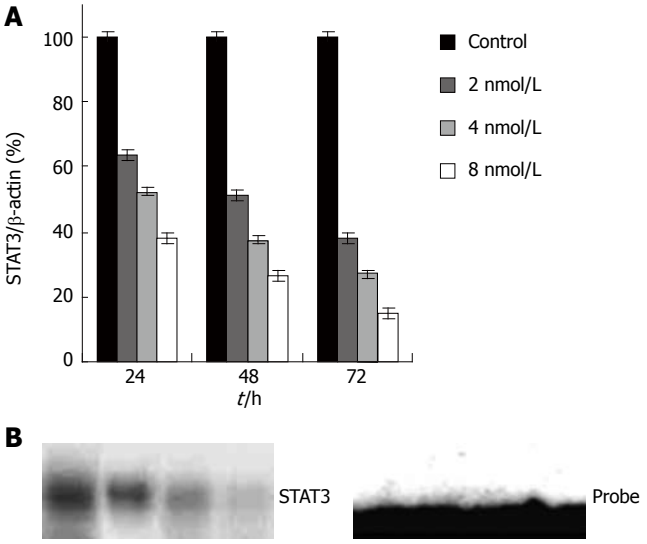


Figure 1 **A**: Expression of STAT3 protein in HT29 cells; **B**: STAT3 DNA-binding activity by EMSA in HT29 cells. 1: Control; 2, 3, 4: siRNA 2, 4, 8 nmol/L.

STAT3 RNAi inhibits anchorage-independent growth in colon cancer cells

Next we evaluated the biological effects of STAT3 suppression in colon cancer HT29 cells by using several different types of assays. Colony formation in soft agar is a property closely associated with malignancy[33]. Figure 2 shows that treatment with STAT3 siRNA induced significant anchorage-independent growth inhibition in a dose-dependent manner.

STAT3 RNAi suppresses invasion in colon cancer cells

Next, we assessed whether STAT3 suppression might decrease the ability of cancer cells to invade the extracellular matrix. HT29 cancer cells were subjected to cell invasion assays, by using Boyden chambers and 8 μm pore size filters coated with Matrigel. Compared with the control group, HT29 cells transfected with siRNA exhibited a scarce ability to cross Matrigel (Figure 3). This difference was statistically significant as compared with control groups ($P < 0.01$).

Effects of STAT3 suppression on anoikis

To explore the molecular mechanisms of invasion of colon cancer, anoikis was studied. To investigate the effect of STAT3 siRNA on anoikis, HT29 cells were subjected to plates seeded in poly-HEMA. After transfection for 48 h,

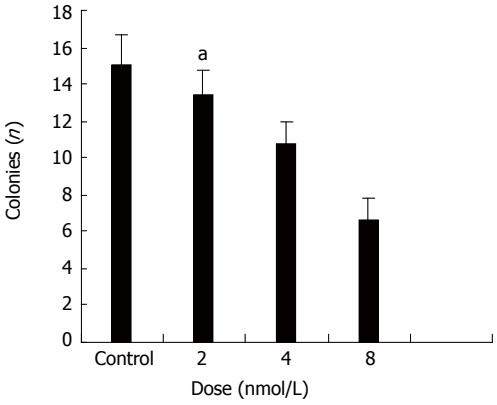


Figure 2 Effects of STAT3 siRNA on anchorage-independent growth of HT29 cells. It shows that treatment with STAT3 siRNA inhibit anchorage-independent growth in a dose-dependent manner. $^{a}P < 0.05$ between siRNA 8 nmol/L and control groups.

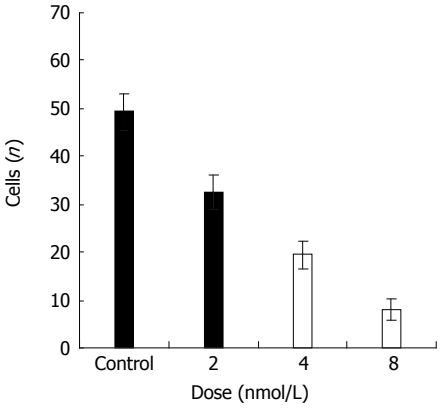


Figure 3 Effects of STAT3 suppression on the invasion ability of HT29 cells.

all cells were removed to plates seeded in poly-HEMA for 12 h. TUNEL and DNA ladder were performed to evaluate the anoikis of HT29 cells. There was little apoptosis in all cancer cells in attached culture. When in a suspended culture, there was significant apoptosis in cancer cells treated with STAT3 siRNA. A significantly higher percentage of apoptotic index was observed in STAT3 siRNA treated cells than in only oligofectamine treated cells (Figure 4A). Consistent with this observation, agarose electrophoresis analysis of HT29 cancer cell extracts showed that there was DNA ladder in cells treated with STAT3 siRNA (Figure 4B). Together these data indicate that siRNA-mediated suppression of the STAT3 gene reduces resistance to anoikis of HT29 cells.

Knockdown of STAT3 expression down regulates survival genes

Recent data indicate that some genes, especially anti-apoptotic genes, contribute to regulation of anoikis[34]. Constitutive activation of STAT3 induces the expression of a number of anti-apoptotic genes including Bcl-xL, a member of the Bcl-2-family of anti-apoptotic genes[20,35], and survivin, a member of the IAP, inhibitors of apoptotic proteins family[36]. Moreover, both of these genes are expressed in colon cancer[37,38]. In order to determine

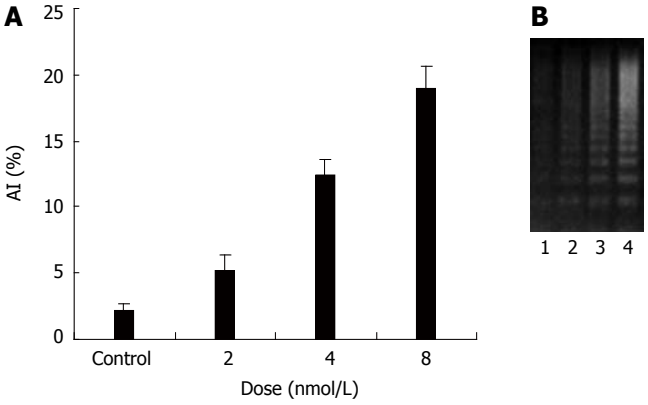


Figure 4 Effects of STAT3 siRNA on anoikis of human colon cancer cell line HT29. **A**: AI (Apoptosis index) induced with STAT3 siRNA in HT29 cells; **B**: DNA ladder by STAT3 siRNA in HT29 cells. 1: Control; 2, 3, 4: siRNA, 2, 4, 8 nmol/L.

whether these two genes might be involved in the STAT3 mediated anoikis resistance in colon cancer, western blot analyses were performed to examine the expression levels of the two genes. Western blot analysis showed that Bcl-xL and survivin protein levels were drastically reduced upon treatment with STAT3 siRNA. Thus, these data show that STAT3 regulates anoikis of at least two distinct antiapoptotic genes in colon cancer HT29 cell (Figure 5).

DISCUSSION

STAT3 is activated by a number of cytokines and growth factors and has diverse functions during embryogenesis and early development[12-14]. Some studies showed that there is persistent activation of STAT3 in a number of human solid tumors including colon cancer[16-18]. Elevated STAT3 activity has been shown to render cells resistant to apoptosis, and inhibition of STAT3 signaling in a number of tumor cell lines with some ways causing a decrease in cell viability and subsequent apoptosis[16,17]. Lots of studies have demonstrated that the transformation process induced by diverse oncogenic protein tyrosine kinases is dependent on STAT3 activation[18,39,40]. Recently it has been shown that STAT3 is constitutively activated in colon cancer. However, the relationships of STAT3 with invasion and metastasis in colon cancer cells remain unclear.

In order to determine the role of STAT3 in colon cancer directly, we have used RNAi to specifically knock down the expression of STAT3, studied the effects of anchorage-independent growth and invasion in the colon cancer cell line HT29. SiRNAs are short oligonucleotides of 21-23 nucleotides in length that can be used *in vitro* to produce sequence specific gene silencing of mammalian cells[32]. It has been shown that siRNAs can be used effectively *in vivo* to suppress gene expression in adult mice[41,42]. SiRNAs can be directly introduced into the CNS to reduce endogenous gene expression[43]. Transfection of colon cancer HT29 cells with STAT3 siRNA resulted in colonies forming membranes with reduced biochemical changes indicative of invasion. In addition, consistent with the results from HT29 cancer cell, SW480 and SW620 cancer cells transfectd with the STAT3 siRNA led to a decrease of both invasion and anchorage-independent

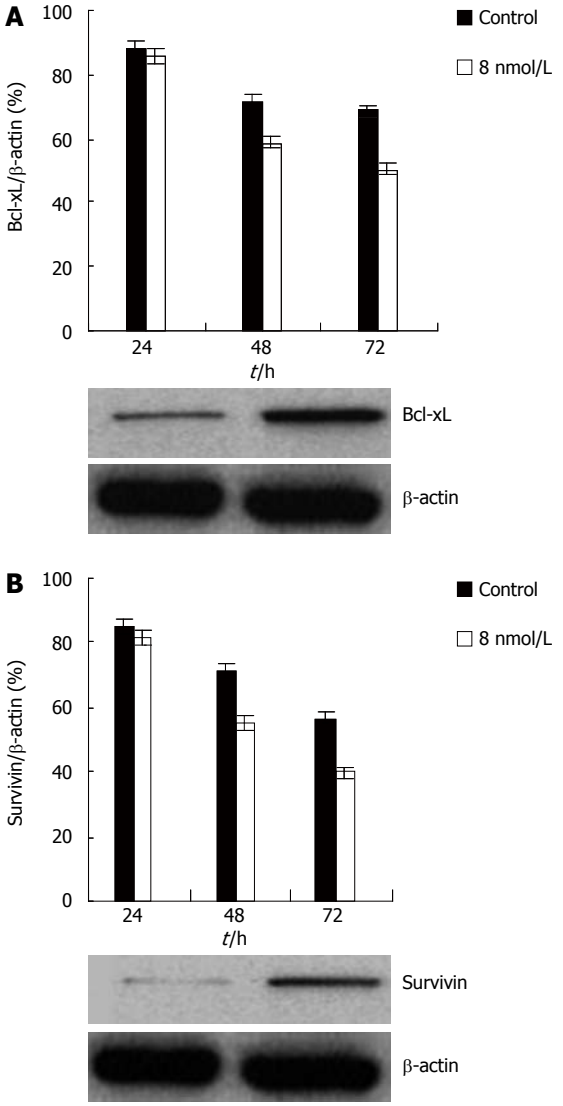


Figure 5 Knockdown of STAT3 expression downregulates Bcl-xL and survivin protein level in the HT29 human colon cancer cell line. **A**: Inhibition of transfection with STAT3 siRNA on Bcl-xL expression of colon cancer HT29 cell; **B**: Suppression of transfection with STAT3 siRNA on survivin expression of colon cancer HT29 cell.

growth. These results are similar to reports previously observed with AG490 inhibiting STAT3 in pancreatic cancer cells[34]. All data suggest that STAT3 down-regulation by RNAi inhibit metastasis of human cancer cell *in vitro*.

The metastasis and invasion of cancer cells were involved in a lot of mechanisms. Anoikis resistance was thought to be high. Anoikis is a peculiar form of apoptosis that is induced by disruption of the interactions between epithelial cells and extracellular matrix[29]. Induction of apoptosis upon loss of anchorage has been termed anoikis (Greek for homelessness)[30]. Anoikis can be considered as a safety program for maintaining normal cell and tissue homeostasis, which prevents survival and reattachment of detached cells to new matrices at inadequate locations. Anoikis has been suggested to act as a physiological barrier to metastasis; resistance to anoikis may allow survival of cancer cells during systemic circulation, thereby facilitating secondary tumour formation in distant organs. Gaining anoikis resistance or anchorage-independent survival is a hallmark of oncogenic transformation. For these experiments, cells are usually seeded in tissue culture dishes that are coated with poly-HEMA, which does not allow cells to attach by preventing matrix deposition. Tumor cells are usually resistant to anoikis[30]. Our results here showed that, in attached culture, neither control cells nor transfected cells exhibited significant apoptosis, suggesting HT29 cells are resistant to anoikis. When seeded in poly-HEMA plates, compared to the control group, there were significant signs of apoptosis in cancer cells treated with STAT3 siRNA. It was shown that clear DNA ladder, the augmented the apoptosis index. These results suggest that STAT3 siRNA induces anoikis of HT29 colon cancer cells.

One mechanism by which STAT3 participates in tumorigenesis is by inhibiting apoptosis through the induction of anti-apoptotic genes such as Bcl-2, Bcl-xL, and Mcl-1[18]. STAT3 responsive elements are found in the promoter region of these genes, suggesting that they are directly regulated by STAT3[16,36]. Interestingly, some reports showed that some anti-apoptotic genes regulate anoikis. Biochemical studies have shown that Bcl-xL, a member of the Bcl-2 family of proteins, induce anoikis of some cancer cells[34,45], and we have found survivin gene is overexpressed in gastric cancer and survivin siRNA reduced the resistance of anoikis of gastric cancer cells (data not shown). In the present study, we have found that both Bcl-xL and survivin are expressed in HT29 cells. Treating HT29 cells with STAT3 siRNA significantly reduces expression levels of both of these genes. These findings suggest that induction of Bcl-xL and/or survivin gene by constitutively activated STAT3 contributes to regulating anoikis of colon cancer cells.

Data presented in this paper are consistent with the growing body of evidence suggesting STAT3 may be an ideal therapeutic target in tumors including colon cancer. We are the first to report that STAT3 siRNA can suppress invasion of colon cancer through inducing anoikis. These results suggest that siRNA may become a useful clinical tool in the future. Since STAT3 signaling is important for the survival of a number of human tumors, STAT3 siRNA could become an effective therapeutic agent for STAT3 dependent tumors.

COMMENTS

Background

Some reports showed that STAT3 has a significant association with tumor invasion and metastasis of a few of cancers, however, the role and mechanism of STAT3 signaling in metastasis of colon cancer remain elusive.

Research frontiers

Induction of apoptosis upon loss of anchorage has been termed anoikis, and anoikis has been suggested to act as a physiological barrier to metastasis. Here we will show that knockdown of STAT3 expression by siRNA inhibit invasion ability by reducing the resistance anoikis in colon cancer cells. In addition, down-regulation of Bcl-xL and/or survivin by STAT3 siRNA might play an important role in inducing anoikis in human colon cancer cell.

Innovations and breakthroughs

The paperwork showed that that STAT3 siRNA can inhibit invasion ability of colon cancer cells through inducing anoikis, and suppression of Bcl-xL and/or survivin by STAT3 siRNA might contribute to regulation of anoikis of human colon cancer cell.

Applications

Here we will show that knockdown of STAT3 expression by siRNA inhibits invasion ability and reduces the resistance anoikis in colon cancer cells. The paper would help to clarify the mechanism of invasion and metastasis of colon cancer and improve the choice of therapeutic strategy.

Terminology

Anoikis (Greek for homelessness) is a peculiar form of apoptosis that is induced by disruption of the interactions between epithelial cells and extracellular matrix.

Peer review

This is an interesting paper investigating the *in vitro* effect and mechanisms of STAT3 siRNAs in a colorectal cancer (CRC) cell line. The main finding of the study was that STAT3 siRNA can inhibit the invasion ability of colon cancer cells through inducing anoikis, in addition antiapoptotic genes survivin and Bcl-xL may contribute to regulation of anoikis.

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BASIC RESEARCH

Effect of Daxx on cholesterol accumulation in hepatic cells

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Abstract

AIM: To study the effect of Daxx on cholesterol accumulation in hepatic cells.

METHODS: Sprague Dawley (SD) rats were fed a normal or high fat diet for 6 wk, and serum lipids and Daxx expression of hepatic tissues were measured by immunoblot assays. $HepG_2$ cells were transfected with the pEGFP-C1/Daxx or pEGFP-C1 plasmid. Cells stably transfected with Daxx were identified by RT-PCR analysis. Total cholesterol levels were determined by high performance liquid chromatography. Activated-SREBP and caveolin-1 were assayed by western blotting.

RESULTS: Hepatic Daxx protein was higher in normal rats than in high fat diet-fed rats. Noticeable negative correlations were seen between Daxx and LDL-C (γ = -7.56, P = 0.018), and between Daxx and TC (γ = -9.07, P = 0.01), respectively. The total cholesterol of $HepG_2$/GFP-Daxx cells was lower than that of control cells or $HepG_2$/GFP cells (9.28 ± 0.19 *vs* 14.36 ± 4.45 or 13.94 ± 2.62, both $P < 0.05$). Furthermore, in $HepG_2$/GFP cells, the expression of activated SREBP was lower than that of control cells, whereas caveolin-1 expression was higher.

CONCLUSION: Overexpression of Daxx in $HepG_2$ cells decreased intracellular cholesterol accumulation, which might be associated with inhibition of SREBP activity and an increase in caveolin-1 expression.

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Key words: Daxx; Cholesterol; Hepatic cells; Sterol regulatory element-binding protein; Caveolin-1

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INTRODUCTION

Daxx was first identified as a death-associated protein capable of binding the cytosolic domain of fas, an apoptosis-inducing member of the tumor necrosis factor (TNF) receptor family[1]. Daxx co-localizes with Promyelocytic Leukemia Protein (PML) within nuclear promyelocytic oncogenic domains (PODs)[2,3]. PML and/or POD-associated proteins may function as an important cofactor in governing nuclear hormone receptor transcriptional activity and function[4,5]. Recent studies[6,7] implied Daxx could negatively modulate androgen receptor (AR) transcriptional activity. Androgens affect lipogenic gene expression not only in tumor cells, but also in normal androgen target tissues *in vivo*[8]. AR can directly upregulate sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP) by binding an androgen response element in intron 8 of the SCAP gene[9]. Activated SREBP can increase the mRNA and protein levels of genes involved in fatty acid (fatty acid synthase and acetyl-CoA-carboxylase), and cholesterol synthesis (HMG-CoA-reductase and farnesyl diphosphate synthase)[10]. These results indicate that Daxx could possibly regulate cellular cholesterol metabolism by the SREBP pathway.

In the present study, we investigated the correlations between Daxx expression and cholesterol accumulation in liver cells. The findings herein show that overexpression of Daxx in $HepG_2$ cells may decrease intracellular cholesterol, which may be associated with inhibition of SREBP activity related to cholesterol synthesis and an increase in caveolin-1 expression related to excretion.

MATERIALS AND METHODS

Materials

Modified Eagle medium (MEM) and fetal bovine serum

were purchased from Gibco BRL. An antibody (Santa Cruz) directed against active SREBP was used to detect the activation of SREBP, and polyclonal anti-Daxx antibody or anti-caveolin-1 antibody (Santa Cruz) was used to assay the respective protein expression. The plasmids of pEGFP-C1/Daxx and pEGFP-C1 were gifts from Dr. Yanping[11]. The pEGFP-C1/Daxx contains a full-length cDNA of hDaxx in pEGFP-C1 vector. All reagents were of analysis grade.

Animal and diets

Male Sprague Dawley (SD) rats (210 g ± 10 g) were obtained from the animal laboratory of Nanhua University. The animals were individually housed in plastic cages in a temperature (23℃ ± 2℃) and light (alternating 12 h periods of light and dark) controlled room. The rats were randomly divided into two groups. The control group was fed a normal diet, and another group was fed a high-fat diet (15%, lard, wt/wt, HFD) for 6 wk. Rats were allowed free access to food and deionized water throughout the test period. At the end of the experiment, the rats were anesthetized with ketamine and injected with 150 IU of heparin per kilogram of body weight. Fifteen minutes later, the rats were sacrificed by disarticulation. Blood samples were taken from the neck into glass tubes, and serum was obtained by centrifugation (2000 × *g* for 10 min at 4℃). The livers were removed and rinsed with physiological saline. All samples were stored at -70℃ until use.

Estimation of serum lipids

For determination of serum total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), triacylglycerol (TG) concentration, and high density lipoprotein-cholesterol (HDL-C), the corresponding diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used according to the manufacturer's instructions.

Cell culture and transfections

$HepG_2$ cells, a human hepatocyte cell line, were obtained from Zhong Shan University (Guangzhou, China). The cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin, at 37℃ in a humidified incubator containing 5% CO_2. Cells were seeded at a density of 1×10^5 cells/well in a 24-well plate and cultured for 24 h to 60%-80% confluency. To obtain stable transfectants, $HepG_2$ cells were transfected with the pEGFP-C1/Daxx or pEGFP-C1 plasmid using Lipofect 2000 Plus reagent (Invitrogen) in serum-free medium for 4 h at 37℃, according to the manufacturer's recommendations. The transfection medium was removed, and fresh complete growth medium was added. After 24 h post-transfection, the cells in two wells were split into 10-cm dishes in a medium containing 500 μg/mL geneticin (G418; Amresco, Solon, USA), and the medium was changed every 3 d until G418-resistant colonies were clearly evident. Individual colonies were transferred into 6-well plates to continue incubation with G418 selection medium. Individual colonies were evaluated for Daxx expression by Immunofluorescent Microscopy, and a monoclonal cell line was used for all experiments successively.

Reverse transcription-PCR

Total RNA was extracted from the cells using Trizol reagent (Gibco BRL) according to the manufacturer's protocol. Three micrograms of total RNA were used for reverse transcription in a total volume of 20 μL with the SuperScript preamplification system (Promega, Madison, MI). Aliquots of 2 μL cDNA were subsequently amplified in a total volume of 25 μL using the GeneAmp PCR kit (Promega) following conditions recommended by the manufacturer. The sense and antisense primers for Daxx were 5'-TGGCGCTCTATGTGGCAGAGATC-3' and 5'-CTGCATCTGTTCCAGATCCTCCT-3' (829 bp); the sense and antisense primers for actin that were used as an internal control were 5'-GGTGGCACCTGTGGTCCACC T-3' and 5'-CTTCACTTGTGGCCCAGATAG-3' (420 bp), respectively. The cycling conditions were as follows: 94℃ for 5 min, followed by 28 cycles of 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 1 min, and a final extension of 72℃ for 10 min. PCR products were separated on the 1.5% agarose gel viewed by ethidium bromide staining. These data were acquired with Alpha Imager 2200 software.

Lipid analysis by high performance liquid chromatography (HPLC)

Cells were scraped from culture flasks into 0.9% NaCl (1 mL per 50 cm^2 flask) and homogenized by sonication for 10 s on ice. The protein concentration of cell lysate was determined by a bicinchoninic acid (BCA) kit. An equal volume of freshly prepared cold (-20℃) KOH in ethanol (150 g/L) was added. The cell lysate was repeatedly vortexed until clear. An equal volume of hexane-isopropanol 3:2 (v/v) was then added. The mixture was vortexed for 5 min, followed by centrifugation at 800 × *g* (15℃ for 5 min). The extraction procedure was repeated twice. The combined organic phase was transferred to clean tapered glass tubes and thoroughly dried under nitrogen at 40℃. The tubes were allowed to cool to room temperature. One hundred μL of isopropanol-acetonitrile 20:80 (v/v) was added. The sample was solubilized in an ultrasound water bath at room temperature for 5 min. After centrifugation at 800 × *g* for 5 min, the samples were introduced into the HPLC device using an Agilent 1100 series. Cholesterol was eluted at a flow rate of 1 mL/min, temperature of 40℃ using an eluent consisting of isopropanol-acetonitrile 20:80 (v:v), and detected by UV-absorption at 206 nm[12].

Western blot analysis

Liver tissues excised from rats were analyzed by western blot with an antibody directed against Daxx. $HepG_2$ cells were washed with PBS, and then 0.5 mL of TME lysis buffer (10 mmol/L Tris, pH 7.5, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, and 25 mmol/L NaF) containing fresh 100 μmol/L Na_3VO_4, 20 μg/mL leupeptin, 1 μg/mL pepstatin A, 4 μg/mL aprotinin, and 1 mmol/L DTT were added. Cell lysates were prepared by freezing and thawing of the cells on ice, and subsequent scraping and sonicating for 30 s. The cell lysates were centrifuged for 30 min at 15 000 × *g*. Protein concentrations in the supernatants were determined by a BCA protein assay kit, and the samples were stored at -80℃. For western blot analysis, 20 μg of protein was

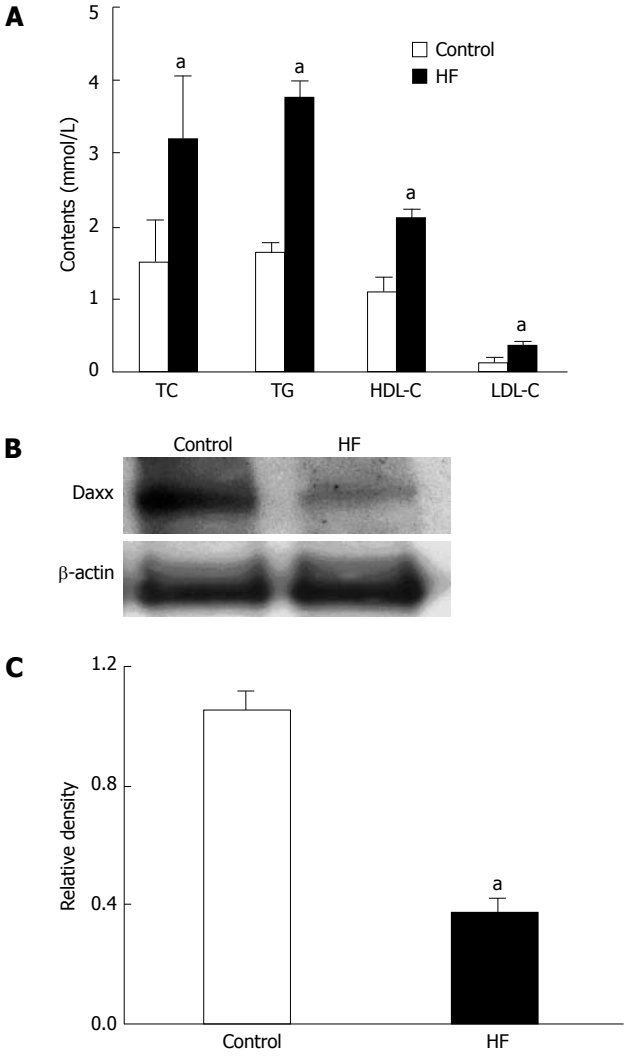


Figure 1 The correlation between Daxx expression of Hepatic tissues and serum cholesterol. **A**: The effect of control or high fat (HF) food on serum cholesterol and triglycerides in rats; **B**: Hepatic Daxx expression of rats as estimated by western blotting; **C**: Quantitative data of Daxx expression, results were normalized to β-actin. Data are the mean ± SE of three independent experiments. Control: normal food. HF: Added high fat to normal food. TC: Total cholesterol; TG: Triglyceride; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol. $^{a}P < 0.05$ *vs* control.

subjected to SDS-PAGE under reducing conditions, and proteins were then transferred to polyvinylidene difluoride membrane as described previously[13]. The membrane was blocked for 2 h at room temperature with a commercial blocking buffer from Life Technologies, Inc. The blots were incubated for 1 h at room temperature with the respective primary antibody (1:2000 dilution), which was followed by 1 h incubation with a secondary antibody (horseradish peroxidase-conjugated, 1:4000 dilution). Target proteins were visualized by a chemiluminescent assay (Amersham-Pharmacia Biotech).

Statistical analysis

The values are expressed as the mean ± SE. The correlation between cholesterol of serum and Daxx was analyzed by SPSS. Statistical analysis of the data was performed using student's *t* test or ANOVA. Values with $P < 0.05$ were considered statistically significant.

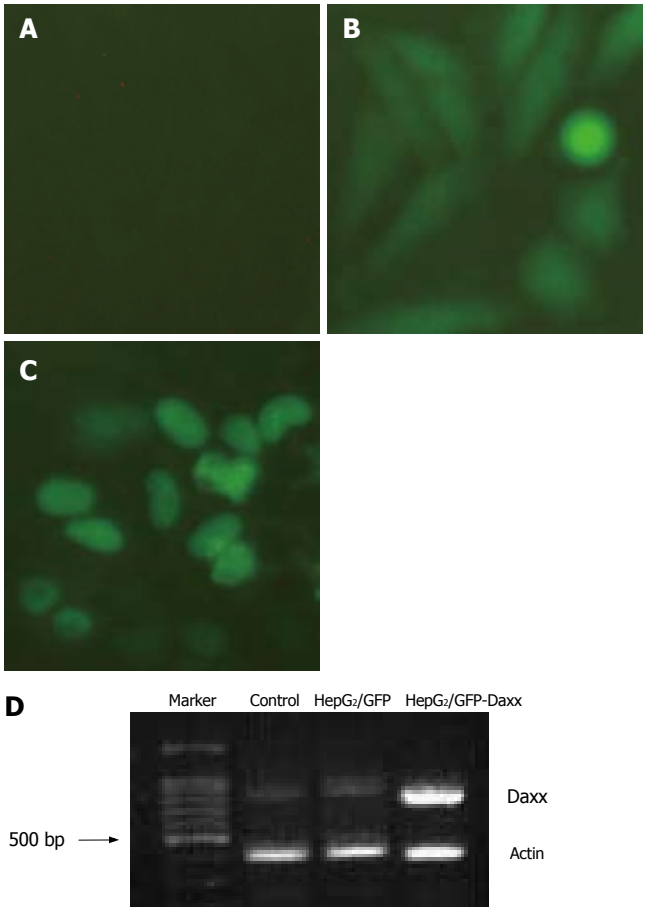


Figure 2 Daxx expression in HepG2 hepatocytes. Cultured HepG2 cells were untransfected (control, **A**) or transfected with pEGFP-C1-Daxx or pEGFP-C1 vectors (**B**, **C**). Images show the location and expression of Daxx in HepG2 cells, which were taken at 400 × magnitude. (**D**) RT-PCR of Daxx mRNA expression.

RESULTS

Correlation of Daxx and cholesterol

HFD feeding for 6 wk resulted in the development of hyperlipidemia in experimental rats, as shown in Figure 1A. Significant increases in TC (211%), TG (231%), HDL-C (197%), and LDL-C (246%) contents were observed in HFD-fed rats compared with those in control rats. At the same time, hepatic Daxx expression in the HFD-fed rats was decreased to one third of the control (Figure 1B and C). These data suggested that Daxx might have possible association with the change of blood lipid content as determined by correlation analysis. Further analysis revealed that there were negative correlations between Daxx and LDL-C (γ = -7.56, P = 0.018) and between Daxx and TC (γ = -9.07, P = 0.01), respectively.

Location and expression of Daxx in HepG2 hepatocytes

HepG2 cells were transfected with pEGFP-C1-Daxx or pEGFP-C1 plasmid. Daxx was mostly located in the nucleus of HepG2 cells (Figure 2A). Because the efficiency of transient-transfection was low, we screened out G418-resistant colonies. The majority of the colonies had fluorescence. PT-PCR analysis indicated that Daxx mRNA expression was significantly increased in HepG2/GFP-Daxx cells when compared with control or HepG2/GFP cells (Figure 2B).

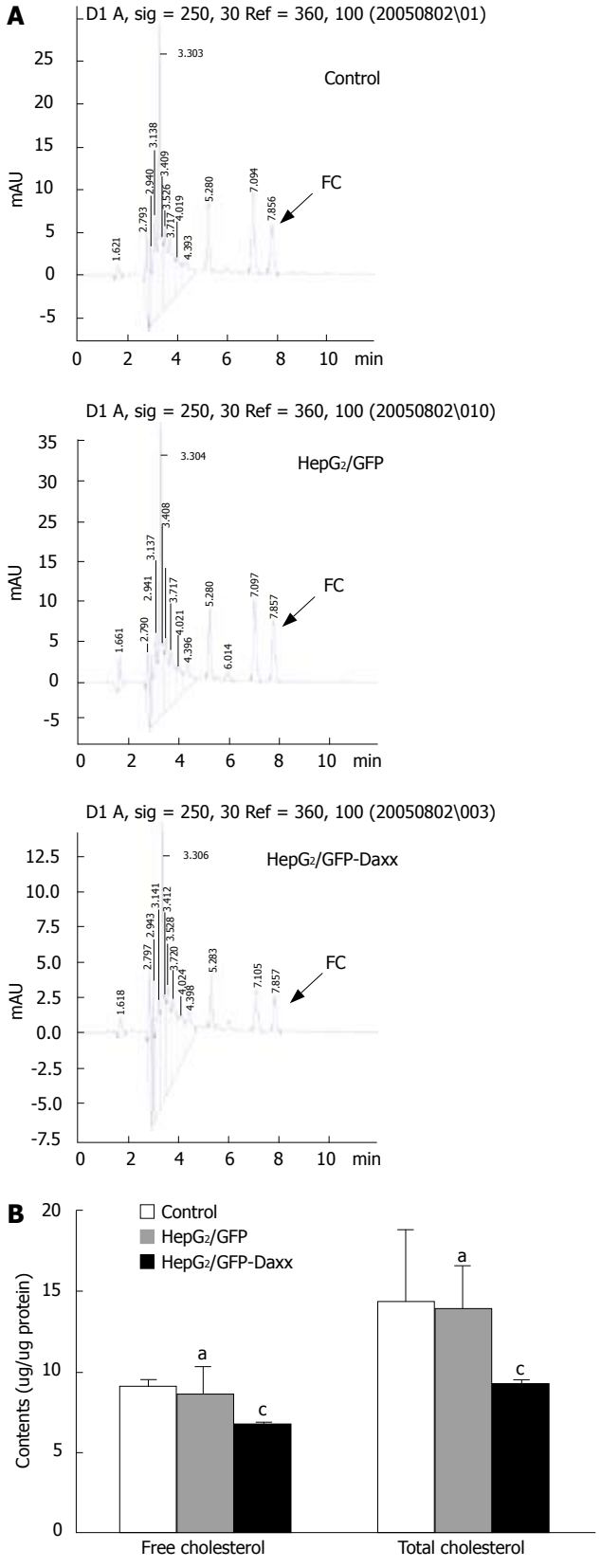


Figure 3 Effect of Daxx overexpression on cholesterol accumulation in HepG2 hepatocytes. **A**: Representative change of intracellular cholesterol levels in HepG2 cells, as determined by HPLC; **B**: The contents of free and total cholesterol in HepG2 cells. FC: Free cholesterol. [a]*P* > 0.05 *vs* control; [c]*P* < 0.05 *vs* control or HepG2/GFP cells.

Effect of Daxx on cholesterol content in HepG2 hepatocytes

Figure 3 shows the effect of Daxx on cholesterol concentration in HepG2 cells as analyzed by HPLC. The arrows show the area of apices which represent the contents of free cholesterol. The area of apices before the arrow represents the total cholesterol (Figure 3A). There was a significant decrease ($P < 0.05$) of the cholesterol concentration in HepG2/GFP-Daxx cells compared with other samples. The free cholesterol in HepG2/GFP-Daxx cells was 6.74 ± 0.13 (μg/μg protein), whereas those of control and HepG2/GFP cells were 9.21 ± 0.37 and 8.66 ± 1.72, respectively. The total cholesterol in HepG2/GFP-Daxx cells was lower than that of control or HepG2/GFP cells (9.28 ± 0.19 *vs* 14.36 ± 4.45 or 13.94 ± 2.62, both $P < 0.05$). The empty vectors did not show obvious effects on cholesterol concentrations in HepG2 cells (Figure 3B).

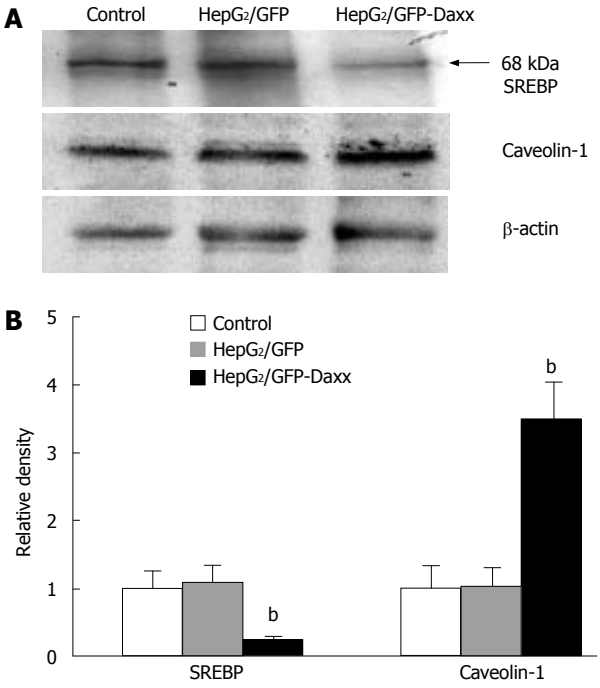


Figure 4 Effect of Daxx on the expression of SREBP and caveolin-1 proteins in HepG2 hepatocytes. **A**: Representative western blot data showing the effects of Daxx on SREBP and caveolin-1 proteins in HepG2 cells; **B**: Quantitative data of the Daxx effect on SREBP and caveolin-1 expression. Data are the mean ± SE of three independent experiments. [b]*P* < 0.01 *vs* control or HepG2/GFP cells.

Effect of Daxx on SREBP and caveolin-1 protein expression in HepG2 hepatocytes

Overexpression of Daxx in HepG2 cells significantly decreased the expression of activated-SREBP from 1 ± 0.23 to 0.21 ± 0.05. Likewise, caveolin-1 expression increased nearly 3.5 times compared to the control (1 ± 0.31 to 3.48 ± 0.56). The empty vectors did not show any effect on the proteins of HepG2 cells (Figure 4A and B).

DISCUSSION

In the animal experiment, we observed that expression of Daxx in hepatic tissues was negatively correlated with hyperlipidemia. The liver is a very important organ for maintaining the physical balance of lipids, and has many proteins (such as SREBP and caveolin) responsible for mediating cholesterol synthesis and excretion[14-16].

It has been reported that hepatic cells predominantly express Daxx[17], but the direct relation between Daxx and cholesterol still remains unclear. Recently, Daxx has been shown primarily to function as a transcriptional regulator[18-20]. These results indicate that Daxx could possibly affect cholesterol accumulation in hepatic cells.

In cultured $HepG_2$ cells, Daxx overexpression decreased the levels of FC and TC compared to those untransfected or transfected with GFP, which indicated that Daxx could affect cholesterol homeostasis of hepatic cells. HMG-CoA-reductase is a key enzyme of cholesterol synthesis and is regulated by sterol regulatory element binding proteins (SREBPs)[21,22]. SREBP-1 represents an important protein of the transcription regulator family (SREBP-1a, -1c, and -2) controlling lipid homeostasis in cells[23,24]. SREBP-1 are synthesized as 125-kDa inactive precursor proteins, and inserted into the membranes of the endoplasmic reticulum where they form tight complexes with SCAP. SREBP is proteolytically cleaved and activated by SCAP when the complex translocates to the Golgi apparatus[25]. The active 68-kDa SREBP fragment migrates to the nucleus and increases the transcription of sterol-responsive element (SRE) that contains many genes encoding lipogenic enzymes belonging to the pathways of cholesterol synthesis[26]. Daxx can inhibit AR transcriptional activity[6,7], which can down-regulates the activity of SCAP[9]. Thus, we checked the activity of SREBP-1 and detected that Daxx has the potential to decrease the expression of active SREBP-1. This also revealed that Daxx might mediate intracellular cholesterol accumulation by inhibiting cholesterol synthesis.

SREBP-1 represses caveolin expression by the SRE/SREBP pathway. In this case, SREBP inhibits *caveolin* gene transcription in contrast to its stimulating effect on other gene promoters[27,28]. Caveolin-1, a type of free cholesterol-binding protein, is another significant protein involved in cholesterol homeostasis[29]. Transfection of cells with full-length caveolin-1 cDNA resulted in the expression of morphologically authentic caveolae structure and FC efflux[30]. The expression of caveolin may represent a mechanism, by which FC excretion became facile[31]. The results of experiments showed that Daxx promoted the expression of caveolin-1. These findings show the possibility of Daxx mediating intracellular cholesterol accumulation presumably by increasing cholesterol excretion.

In conclusion, our results confirm that Daxx is likely to decrease the intracellular cholesterol accumulation by regulating cholesterol synthesis and excretion. One of the main future challenges will be the generation of suitable animal models to be used to dissect Daxx function in cholesterol homeostasis.

COMMENTS

Background

Death-associated protein (Daxx) could negatively modulate androgen receptor (AR) transcriptional activity. Androgens affect lipogenic gene expression not only in tumor cells but also in normal androgen target tissues *in vivo*. AR can directly upregulate sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP) by binding an androgen response element in intron 8 of SCAP gene. Activated SREBP can increase the mRNA and protein levels of genes involved in fatty acid, and cholesterol synthesis. These results indicate that Daxx could possibly regulate cellular cholesterol metabolism by the SREBP pathway.

Research frontiers

Hypercholesrerolemia is mainly a pathologic feature of cardiovascular diseases. The liver is a very important organ for maintaining the physical balance of cholesterol. In liver SREBPs are very important proteins which have been responsible for mediating cholesterol metabolism. Some studies have shown Daxx can regulate SREBP indirectly, but no evidences have suggested Daxx can affect cholesterol balance.

Innovations and breakthroughs

In the present study, we investigated the correlations between Daxx expression and cholesterol accumulation in liver cells. The findings herein show that overexpression of Daxx in $HepG_2$ cells may decrease intracellular cholesterol, which may be associated with inhibition of SREBP activity related to cholesterol synthesis and increase in caveolin-1 expression related to excretion.

Applications

Our study will provide academic value in finding a new function of Daxx, and experimental reference for clinical treatment of hypercholesterolemia.

Peer review

The authors demonstrated the inverse relationship between Daxx expression and cholesterol accumulation in hepatocytes. It appears that the effect of Daxx on cholesterol level in liver cells may be associated with inhibition of SREBP activity and an increase in caveolin-1 expression. The study was well performed and the data are clearly presented.

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BASIC RESEARCH

Experimental study on operative methods of pancreaticojejunostomy with reference to anastomotic patency and postoperative pancreatic exocrine function

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Abstract

AIM: To assess the patency of pancreaticoenterostomy and pancreatic exocrine function after three surgical methods.

METHODS: A pig model of pancreatic ductal dilation was made by ligating the main pancreatic duct. After 4 wk ligation, a total of 36 piglets were divided randomly into four groups. The piglets in the control group underwent laparotomy only; the others were treated by three anastomoses: (1) end-to-end pancreaticojejunostomy invagination (EEPJ); (2) end-to-side duct-to-mucosa sutured anastomosis (ESPJ); or (3) binding pancreaticojejunostomy (BPJ). Anastomotic patency was assessed after 8 wk by body weight gain, intrapancreatic ductal pressure, pancreatic exocrine function secretin test, pancreatography, and macroscopic and histologic features of the anastomotic site.

RESULTS: The EEPJ group had significantly slower weight gain than the ESPJ and BPJ groups on postoperative weeks 6 and 8 ($P < 0.05$). The animals in both the ESPJ and BPJ groups had a similar body weight gain. Intrapancreatic ductal pressure was similar in ESPJ and BPJ. However, pressure in EEPJ was significantly higher than that in ESPJ and BPJ ($P < 0.05$). All three functional parameters, the secretory volume, the flow rate of pancreatic juice, and bicarbonate concentration, were significantly higher in ESPJ and BPJ as compared to EEPJ ($P < 0.05$). However, the three parameters were similar in ESPJ and BPJ. Pancreatography performed after EEPJ revealed dilation and meandering of the main pancreatic duct, and the anastomotic site exhibited a variable degree of occlusion, and even blockage. Pancreatography of ESPJ and BPJ, however, showed normal ductal patency. Histopathology showed that the intestinal mucosa had fused with that of the pancreatic duct, with a gradual and continuous change from one to the other. For EEPJ, the portion of the pancreatic stump protruding into the jejunal lumen was largely replaced by cicatricial fibrous tissue.

CONCLUSION: A mucosa-to-mucosa pancreaticojejunostomy is the best choice for anastomotic patency when compared with EEPJ. BPJ can effectively maintain anastomotic patency and preserve pancreatic exocrine function as well as ESPJ.

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Key words: Pancreaticojejunostomy; Animal model; anastomotic patency; Pancreatic exocrine function; Histopathology; Pancreatography

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INTRODUCTION

Pancreaticoduodenectomy is a well-established operation for resectable periampullary carcinoma. The anastomosis

between the remaining distal pancreas and the gastrointestinal tract after pancreaticoduodenectomy has, however, been the site of complications responsible for considerable morbidity and mortality. Pancreatic leakage is the most common and severe postoperative complication[1-5]. At present, most techniques have been directed toward the prevention of fistulas[3,6-11]. However, long-term anastomotic patency has not been of great importance in the past because of early cancer recurrence and death. Now that the results are improving in both cancer therapy and pancreatic transplantation, the issue of long-term anastomotic patency will grow in importance. Pancreaticoduodenectomy is an unusual gastrointestinal anastomosis in that a hollow muscular organ is being sewn to the side of a solid organ, with a small accentrically placed duct. Therefore, the anastomosis is prone to stenosis and obstruction because of the narrow lumen of the duct and fibrosis of the proximal pancreas after operation. At present, clinical and experimental studies have indicated a better patency rate for duct-to-mucosa anastomosis[12-20]. In theory, the optimum technique is to secure a leakproof anastomosis that will not subsequently produce stenosis and exocrine deficiency. The most important factor affecting the function of the remnant pancreas is the patency of the pancreaticojejunostomy and pancreaticogastrostomy. Data suggest that such stenosis and obstruction often results in eventual exocrine and endocrine dysfunction in animal models and patients[12-20].

Recently, Pen Shuyou *et al* have advocated the use of binding pancreaticojejunostomy (BPJ) after Whipple's resection to minimize pancreatic leakage. Clinical studies have revealed that the present technique is safe, simple and effective in fulfilling current demands on resectional pancreatic surgery[21-23]. So far, few studies appear to have investigated pathophysiological changes induced by BPJ following pancreaticoduodenectomy, particularly in relation to the anastomotic patency and function of the remnant pancreas. In the present study, we attempted to examine anastomotic patency and postoperative pancreatic exocrine function in piglets after three different methods of joining the body and tail of the pancreas to the gastrointestinal tract.

MATERIALS AND METHODS

Materials and animal model for pancreatic duct dilatation (first operation)

Thirty-six domestic piglets of both sexes, weighing 19-21 kg, were obtained from the Animal Center of Zhejiang University. All animals were treated humanely according to the national guidelines for the care of animals. The experiment was approved by the Animal Experiment Committee of the University of Zhejiang. The animals were fasted for 24 h before the experiment and allowed food and water *ad libitum* after the operation. The piglets underwent a preliminary sterile laparotomy under general anesthesia with 25 mg/kg sodium pentobarbital. The main pancreatic duct draining the body and tail was carefully separated and ligated with 1/0 silk thread, which produced total obstruction, and left the duodenal vascular arcade intact, as described by Pitkaranta *et al*[24]. This was then relieved by one of three methods at a second operation. For both operations, the animals were fasted the night before surgery. Intravenous cannulation and endotracheal intubation were performed after anesthetic induction. Animals received 1 L of normal saline during each procedure. Penicillin G benzathine in aqueous suspension (4 MU) was administered intramuscularly before the operation and continued the following day. The operation was performed under strict aseptic conditions. The controls underwent laparotomy only.

Pancreatic anastomosis (second operation)

Four weeks after ligation of the main duct, laparotomy was performed again. After resection of the pancreatic head (5 cm), the diameter of the duct at the cut surface was measured, and the pigs underwent sequentially one of three types of pancreatico-intestinal anastomosis, or were kept as controls. The management of the pancreatic stump was: (1) end-to-end pancreaticojejunostomy invagination (EEPJ) to a Roux-Y loop in nine piglets, using two layers (1/0 silk interrupted inner and outer layer); (2) end-to-side duct-to-mucosa sutured anastomosis to a Roux-Y loop (ESPJ) in nine piglets, using two layers (1/0 silk interrupted inner and outer layer), completed by a second layer between the pancreatic capsula and jejunal seromuscular wall; or (3) binding pancreaticojejunostomy (BPJ) in nine piglets. The surgical techniques have been described previously[21-23]. The main procedure was as follows. End-to-end duct-to-mucosa sutured (1/0 silk interrupted) anastomosis to a Roux-Y loop was performed, the pancreatic duct and jejunal mucosa were sutured, and the stitch was only inserted to the submucosal layer of the jejunal wall (Figure 1A). This procedure was slightly modified from those previously described. Finally, a piece of unabsorbable thread (7/0 silk) was used to bind circumferentially together the jejunal seromuscular sheath and pancreatic remnant (Figure 1B). The thread tie is just tight enough to allow the tip of a medium-sized hemostatic forceps to be passed underneath the ligature. No stent was used in any of the anastomoses. Roux-Y limbs of 45 cm were created using a standard side-to-side technique. After 2 d of water, the animals had free access to standard lab chow. Animals were kept in individual living quarters. Body weight was determined with a cage scale preoperatively and repeated at 2, 4, 6 and 8 wk during follow-up, until the third operation.

Measurement of intrapancreatic ductal pressure

Eight weeks after the second operation, laparotomy was again performed. Intrapancreatic ductal pressure was measured in all animals. As described by Bradley[25], a No 21 scalp vein needle (attached to a short length of connecting tube, a three-way stopcock, a manometer, and a 2-mL syringe, all filled with saline solution) was introduced into the duct of Wirsung directly through the surface of the pancreas. Fluid aspirated from the pancreatic duct into the syringe to confirm the position of the needle was meticulously replaced from the syringe before pressure measurement. The saline manometer was arbitrarily zeroed at the level of the anastomotic site. Fluctuation in pressure was noted with respiration in most animals. All pressures were recorded in the duct of Wirsung before operative pancreatography and surgical manipulation.

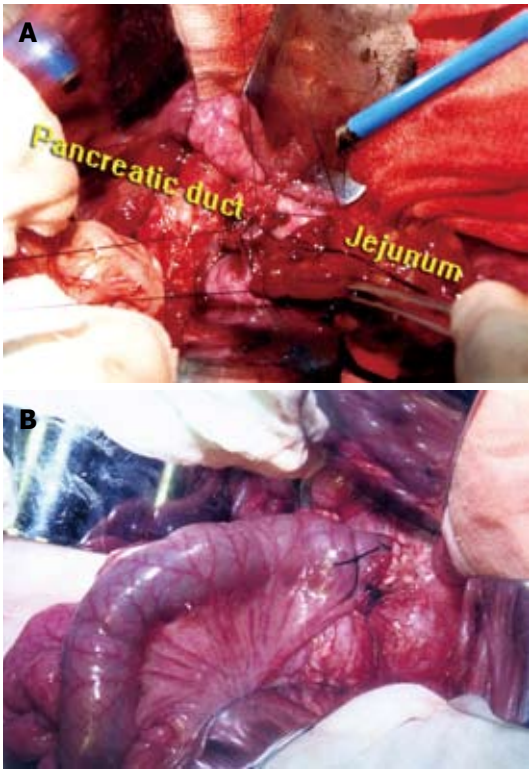


Figure 1 **A**: Pancreatic duct and jejunal mucosa are sutured, and stitch is only inserted to the submucosal layer of the jejunal wall; **B**: A piece of unabsorbable thread (7/0 silk) is used to bind together circumferentially the jejunal seromuscular sheath and pancreatic remnant.

Secretin test and minimal pressure required to obtain flow through the anastomosis

The jejunal wall was incised. The anastomotic site was grossly examined, and the status of the pancreatic drainage flow at the anastomosis was inspected by direct visual observation. Pancreatic exocrine function was evaluated by the secretin test. After 30 min basal secretion collection, secretin was administered (2 U/kg iv bolus; Sigma, St. Louis, MO, USA), and pancreatic juice at the anastomotic site was collected at intervals of 10 min for 60 min. Secretory volume, flow rate of pancreatic juice, and bicarbonate concentration were determined using standard laboratory techniques.

Pancreatographic investigation

After resection of the remnant pancreas and jejunum *en masse*, pancreatography was performed immediately by inserting a catheter into the main duct at the tail of the pancreas. The contrast medium (meglumine diatrizoate) was instilled from a height of 10 cm to equalize the pressure as uniformly as possible. The extracted pancreas was placed on an X-Omat TL film (Eastman Kodak, Rochester, NY, USA) for roentgenography at 300 mA, 26 kVp, 1.0 s and a film-focus distance of 88 cm. The maximum diameter of the main pancreatic duct was measured.

Macroscopic and histologic assessment

The pancreatic duct was examined for patency by cannulating the anastomosis distally, and testing to see if a catheter could be passed freely into the duct. Then, the anastomotic site and the long axis of the pancreatic duct of the body and tail were incised and examined macroscopically, and the specimens were fixed in 10% neutral formalin for 3 d. Sections containing the anastomotic site were prepared, embedded in paraffin, and stained with hematoxylin and eosin for histologic evaluation.

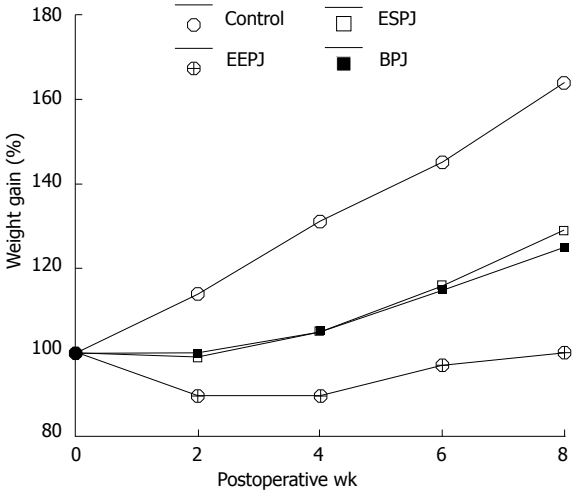


Figure 2 Percentage weight gain in pigs undergoing three types of pancreatoenteric anastomosis and in control pigs.

Statistical analysis

Data are presented as mean ± SD unless otherwise indicated. Statistical significance was assessed by analysis of variance or Student's *t* test where appropriate. Results were considered significant at $P < 0.05$.

RESULTS

Mean body weight changes

A percentage body weight change in relation to the preoperative value was determined for each animal. The postoperative weight gain for each group is shown in Figure 2. Duct-to-mucosa sutured anastomoses (ESPJ and BPJ) differed significantly from controls and EEPJ on postoperative week (POW) 2, 4, 6 and 8 ($P < 0.05$). The animals in the ESPJ and BPJ groups had a similar body weight gain, and these two types of anastomoses did not differ significantly from each other on POW 2, 4, 6 and 8 ($P > 0.05$). These mean body trends, however, suggested that the status of the anastomosis had some physiologic effect.

Intraperitoneal findings

All piglets recovered from the operation, and no fatality was encountered. Atrophy of the pancreatic parenchyma was shown in all animals in the three groups, and the main pancreatic duct at the cut surface of the pancreatic stump was markedly dilated at the end of POW 4 after the first operation (Figure 3, range 3.1-3.5 mm). At the end of POW 8 after the second operation, adhesion of the anastomotic portion to adjacent tissue was detected in all animals, the anastomotic site was healed completely, and no suture failure and intraperitoneal abscess formation

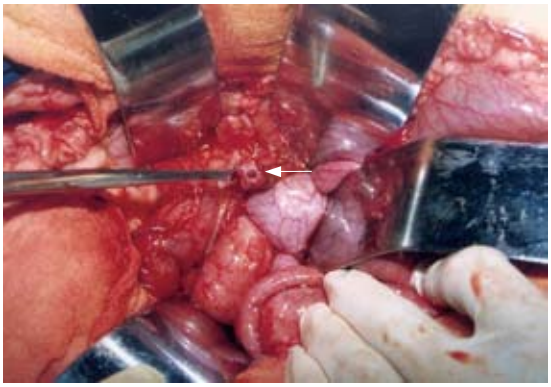


Figure 3 Duct at the cut surface of pancreatic stump was markedly dilated at the end of POW 4 after ligation of the main pancreatic duct.

Table 1 Average intraductal pressure in three types of pancreatic anastomosis and in controls

Group	*n*	Intraductal pressure (cm H_2O)
Control (normal)	9	13.24 ± 1.31
EEPJ	9	32.51 ± 2.19[a]
ESPJ	9	18.41 ± 1.42[a,c]
BPJ	9	19.13 ± 1.72[a,c]

[a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* EEPJ.

Table 2 Pancreatic secretion in three types of pancreatic anastomosis and in controls

Group	*n*	Secretory volume (mL/h)	Flow rate (mL/kg/30 min)	Bicarbonate concentration (mEq/L)
Control (normal)	9	137.56 ± 12.18	1.38 ± 0.13	133.56 ± 13.95
EEPJ	9	34.78 ± 4.82[a]	0.35 ± 0.10[a]	72.78 ± 10.58[a]
ESPJ	9	108.33 ± 10.65[a,c]	1.11 ± 0.15[a,c]	110.44 ± 12.52[a,c]
BPJ	9	101.23 ± 12.87[a,c]	1.05 ± 0.19[a,c]	112.67 ± 17.68[a,c]

[a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* EEPJ.

around the anastomotic site were observed in any animal. None of the piglets showed anastomotic complications.

Intrapancreatic duct pressure

Intrapancreatic duct pressure on POW 8 is shown in Table 1. The levels were similar in ESPJ and BPJ, and no significance was found between the two procedures ($P > 0.05$). However, pressure in EEPJ was significantly higher than that in ESPJ and BPJ ($P < 0.05$).

Pancreatic exocrine function assessed by the secretin test

The results of the secretin test for each group are summarized in Table 2. All three parameters, secretory volume, flow rate of pancreatic juice, and bicarbonate concentration, were significantly higher in ESPJ and BPJ compared to EEPJ ($P < 0.05$). However, the levels of the three parameters were similar in ESPJ and BPJ, with no significant difference between these two techniques ($P > 0.05$). These parameters suggested that the status of the anastomosis had some mechanical effect on pancreatic exocrine function.

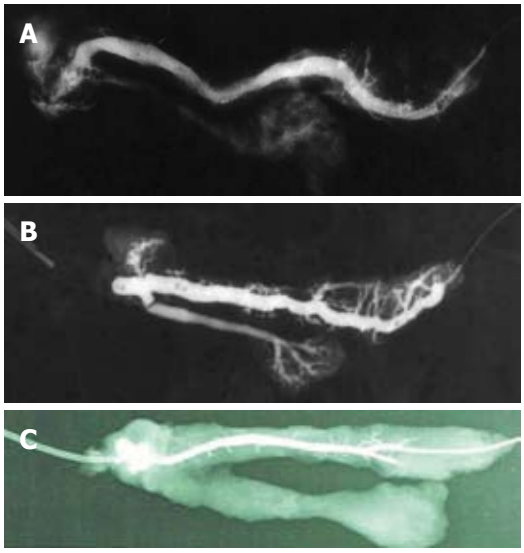


Figure 4 **A**: Anastomotic site was incompletely obstructed; **B**: Duct was blocked in the anastomotic region; **C**: Normal ductal patency without stenosis at the anastomotic site and without ductal dilatation proximal to it.

Pancreatography and maximum diameter of the main pancreatic duct

Pancreatography performed after EEPJ revealed dilation and meandering of the main pancreatic duct, together with indistinct depiction of the secondary and tertiary bifurcation of the duct, and the anastomotic site exhibited a variable degree of occlusion (Figure 4A). The duct was partially blocked in one animal and completely blocked in another in the anastomotic region (Figure 4B). Pancreatography of both ESPJ and BPJ, however, showed normal ductal patency without stenosis at the anastomotic site and without ductal dilatation and meandering of the main pancreatic duct, but distinct secondary and tertiary bifurcations of the ducts (Figure 4C). In one animal only, a slight dilatation of the anastomosed duct was detected in BPJ. Macroscopically, cicatricial narrowing of the main pancreatic duct was not observed, which suggested effective pancreatic juice drainage. The maximum diameter of the main pancreatic duct is shown in Table 3. Significant differences were found in both the ESPJ and BPJ groups in comparison with the EEPJ group ($P < 0.05$). Main pancreatic duct diameters showed no significant differences between ESPJ and BPJ ($P > 0.05$).

Macroscopic and histologic features of the anastomotic site

Macroscopically, in all specimens, there was firm union at the site of anastomosis, and the pancreas had an atrophic appearance. The specimens removed 8 wk after ESPJ and BPJ clearly showed complete mucosal continuity and good connective tissue union between the pancreatic duct and the jejunal lumen (Figure 5). The results were verified histologically, and microscopy showed that the intestinal mucosa had fused with that of the pancreatic duct, with gradual and continuous changes from one to the other (Figure 6). In contrast, in biopsies obtained in EEPJ, the portion of the pancreatic stump protruding into the jejunal lumen was largely replaced by cicatricial fibrous tissue in

Table 3 Maximum diameter of the main pancreatic duct

Group	*n*	Maximum diameter (mm)
Control	9	2.08 ± 0.33
EEPJ	9	6.13 ± 0.47[a]
ESPJ	9	2.98 ± 0.41[a,c]
BPJ	9	3.02 ± 0.45[a,c]

[a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* EEPJ.

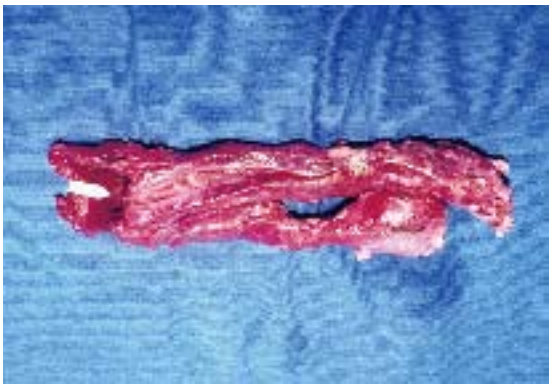


Figure 5 Longitudinal specimen reveals good connective tissue union between the pancreatic duct and the jejunal lumen.

which concentric fibrous bands with scattered mononuclear inflammatory cells were seen, and no intestinal mucosa was observed to cover the remnant cut surface.

DISCUSSION

Since Whipple's introduction of pancreaticoduodenectomy in 1935 for the treatment of cancer of the ampulla of Vater and the pancreatic head, it has become the treatment of choice in the management of periampullary cancer. However, the procedure incurs much criticism because of its high morbidity and mortality. Operative mortality has decreased dramatically over the past decades, due in part to improvement in operative technique and perioperative care[4,5,26]. Additionally, long-term survival has significantly improved and this has engendered a need to consider the importance of postoperative quality of life after resectional pancreatic surgery. The approaches involve the preservation of the exocrine and endocrine function by different techniques of anastomosis between the pancreatic stump and the digestive tract. Most of the proposed techniques are aimed at preventing leakage. Common procedures include EEPJ, ESPJ, and pancreatogastrostomy (which seems to be the least-performed procedure).

It is generally accepted that pancreatic consistency during Whipple resection is generally classified into one of two groups: normal, soft, and friable with non-dilated ducts, or firm and fibrotic with dilated ducts, with the former group having a high susceptibility to anastomotic leakage. The soft nature of the pancreatic remnant, combined with a relatively high pancreatic juice secretion rate through a non-dilated pancreatic duct, is thought to contribute to anastomotic leakage[27]. In many

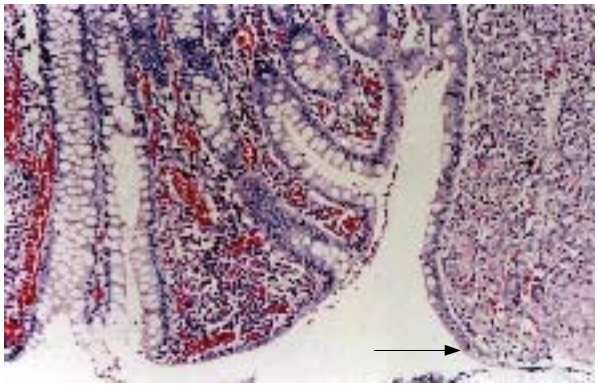


Figure 6 Section showing that the intestinal mucosa had fused with that of the pancreatic duct, with gradual and continuous changes from one to the other. Original magnification × 100.

patients with cancer and pancreatitis, the duct is dilated from obstruction, and the obstructed gland is fibrotic and easier to sew than when normal. Theoretically, the perfect approach is based on a simple general surgical principle: the absence of immediate leakage and the long-term patency of any intestinal pancreatic anastomosis. Previous studies have indicated the most important factor that influences the function of the remaining pancreas after pancreatoduodenectomy is the patency of pancreaticoenterostomy. There is a definite relationship between the degree of duct obstruction and the severity of the exocrine changes[12-20]. Therefore, preserving anastomotic patency may be essential for good remnant pancreatic function.

Tiscornia and Dreiling[28] ligated the main pancreatic duct and studied pancreatic secretion in 12 dogs with Thomas cannulas. After 7-46 d, the ligated segment of the main pancreatic duct was resected, and a primary duct-to-duct anastomosis was created to reestablish flow. Secretion was studied for up to 7 mo, and they found progressive improvement in bicarbonate and enzyme secretion, with eventual return to normal. Through clinical and experimental study, Tanaka and his colleagues[13] have revealed that the exocrine pancreas before pancreaticoduodenectomy was impaired due to obstructive pancreatitis, and that postoperative pancreatic function was well preserved at a level close to that in the control group, even after 50% resection of the pancreas, if pancreatic duct drainage was effectively performed. Several authors[12-20] have pointed out occlusion of the pancreatic duct after pancreaticoduodenectomy, which leads to an insufficiency in pancreatic exocrine and possibly endocrine function in later years. If this happens, the advantages of lower mortality and morbidity immediately after pancreaticoduodenectomy could be offset. It is conceivable that after the Whipple operation with extirpation of the sphincter of Oddi, the only outflow resistance encountered would be that due to stenosis at the site of pancreaticoenterostomy. At present, data obtained from both clinical and experimental studies have indicated that mucosa-to-mucosa pancreaticojejunostomy or pancreaticogastrostomy is the best choice for anastomotic patency[12-20].

Recently, the new method of BPJ has increasingly

been performed, especially in China, although other pancreaticojejunostomy techniques are also common. The clinical and experimental data have demonstrated that it is a safe, simple and efficient technique that avoids the primary complication of anastomatic leakage, whether the pancreatic remnant is soft or hard[21-23]. On the basis of these data, we have modified that approach slightly to test whether the patency of anastomosis and the function of the remnant pancreas of modified BPJ (mBPJ) is likely to be as good as, or better than that of the mucosa-mucosa anastomosis, for which the pancreatic remnant is hard and the pancreatic duct is dilated. To the best of our knowledge, no adequate studies appear to have been carried out.

The study of the patency of pancreaticoenterostomy and function of the remaining pancreas requires a suitable pancreatic duct dilatation model that results in an easier and more reliable experiment, although careful and meticulous anastomosis can be performed with microsurgical techniques. The digestive system of the pig closely resembles that of humans[24], therefore, we chose a duct-ligated model in piglets. This piglet model is reproducible and easy to perform, it produces changes in the pancreas analogous to those usually found in patients undergoing pancreatoduodenectomy, and the conclusion of the study is more convincing. Total obstruction of the main pancreatic duct in the experimental animals resulted in distention of pancreatic ducts and ductules.

In our previous study, we have demonstrated that there is a stronger anastomosis (bursting pressure and breaking strength) in BPJ than in EEPJ, and the anastomotic healing is better and more rapid, thus we conclude that BPJ is a safer and more reliable method than EEPJ, although no comparison has been made between BPJ and ESPJ[29]. However, the principle objectives of this study were to assess the patency of anastomosis, the exocrine function of the remnant pancreas, and the feasibility of mBPJ. The patency of anastomosis of mBPJ was the endpoint of the study.

In our present study, pancreatic duct patency without stenosis at the anastomotic site was confirmed directly by ductography and gross examination in all animals studied by ESPJ and BPJ. Similar patency was found in both ESPJ and BPJ. However, EEPJ revealed dilation and meandering of the main pancreatic duct, which seemed to be caused by pancreatic juice retention, due to poor drainage of pancreatic juice in the anastomosed pancreas. The degree of dilatation of the main pancreatic duct seems to correspond to the likelihood of anastomotic stenosis of pancreaticoenterostomy. The body weight gain (which is positively related to the pancreatic exocrine secretion[30]), secretin test, and intrapancreatic ductal pressure are in agreement with the functional status of the pancreatic duct and anastomotic site. Jejunal mucosa is in continuity with the pancreatic duct at the anastomotic site in ESPJ and BPJ. Consequently, we were able to obtain a very secure patent anastomosis in the piglet model. Our data were in accordance with those of previous studies that reported better patency with mucosa-mucosa anastomosis compared with EEPJ. According to the results of our study, and taking into consideration the advantage of preventing anastomotic breakdown or pancreatic leakage and maintaining patency of the pancreatic duct by BPJ, we believe that the procedure will result in a more anatomically secure anastomosis than that with the standard method, and should decrease the morbidity and mortality of the operation, thus increasing its acceptability as the operation of choice for Whipple resection.

COMMENTS

Background

Resectional pancreatic surgery is carried out with increasing frequency for both malignant and benign diseases. However, no adequate studies appear to have been made of the pathophysiological changes following pancreaticoduodenectomy, particularly of anastomotic patency and function of the remnant pancreas. The most important factor affecting the function of the remnant pancreas is the patency of the pancreaticoenterotomy. In the present study, we attempted to examine anastomotic patency and postoperative pancreatic exocrine function in piglets after three different anastomotic methods using end-to-end pancreaticojejunostomy invagination (EEPJ), end-to-side duct-to-mucosa sutured anastomosis (ESPJ) and binding pancreaticojejunostomy (BPJ) (a new method).

Research frontiers

There is little agreement about the best technique for pancreatic anastomosis of the gastrointestinal tract. The optimum technique is to secure a leakproof anastomosis that will not subsequently produce stenosis and exocrine deficiency. However, few clinical and experimental studies have focused on the postoperative pancreatic exocrine function. The main findings of this study were that the animals undergoing ESPJ and BPJ were much better than those undergoing EEJP, using several parameters of measurement, weight gain, intraductal pressure, secretory volume and flow of pancreatic juice, ductal patency and absence of duct dilatation.

Innovations and breakthroughs

As a new method of anastomosis, BPJ has been proven to be a safe, simple and efficient technique that avoids the primary complication of anastomatic leakage clinically and experimentally. The authors also confirmed that BPJ may effectively maintain anastomotic patency and preserve pancreatic exocrine function as well as ESPJ, and a mucosa-to-mucosa pancreaticojejunostomy is the best choice for anastomotic patency, when compared with EEPJ.

Applications

Our data were in accordance with those of previous studies that have reported better patency with mucosa-mucosa anastomosis when compared with EEPJ. According to our results, and taking into consideration the advantage of preventing anastomotic breakdown or pancreatic leakage and maintaining patency of the pancreatic duct by BPJ, we believe that this procedure will result in a more anatomically secure anastomosis than that with the standard method, and should decrease the morbidity and mortality of the operation, thus increasing its acceptability as the operation of choice for Whipple resection.

Terminology

BPJ, a new method introduced to pancreatic surgery by Pen Shuyou *et al*, has increasingly been performed, especially in China, although other pancreaticojejunostomy techniques are also common. Clinical studies have revealed that the present technique is safe, simple and effective in fulfilling current demands on resectional pancreatic surgery. However, the anastomotic patency and residual exocrine pancreatic function after BPJ continues to require adequate evaluation.

Peer review

The authors are to be commended on their excellent piece of research. There have been few clinical and experimental studies with a focus on the pathophysiological changes of pancreaticoenterotomy, particularly of anastomotic patency and function of the remnant pancreas. This is an interesting study, well designed and well performed. The discussions are to the point and not overstated. The authors' findings certainly merit attention.

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CLINICAL RESEARCH

Management of gastric fundal varices without gastro-renal shunt in 15 patients

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Abstract

AIM: To examine the portal hemodynamics of gastric fundal varices (GV) without gastro-renal shunt (GRS), and to retrospectively investigate the effects of various kinds of treatment on eradication.

METHODS: Ninety-four liver cirrhosis patients at high-risk of GV were treated in our hospital and enrolled in this study. We retrospectively examined their characteristics, liver function, and portal hemodynamics of GV. We performed balloon-occluded retrograde transvenous obliteration (BRTO) at first. If it was not technically possible to perform BRTO, endoscopic injection sclerotherapy using α-cyanoacrylate glue (CA) or percutaneous transhepatic obliteration (PTO) was performed.

RESULTS: Among the 94 patients, a GRS was present in 79 (84.0%), and absent in the remaining 15 (16.0%). The subphrenic vein was connected to the inferior vena cava as the drainage vein in 13 (86.7%) out of the 15 cases without GRS. We performed BRTO in 6 patients, CA in 4 patients and PTO in 5 patients. The eradication rate was 100% for each procedure, but the rate of early recurrence within 6 mo was 16.7% for BRTO, 50.0% for CA and 40.0% for PTO, respectively.

CONCLUSION: We should examine the hemodynamics before treatment of GV irrespective of the existence of GRS. If this hemodynamic examination reveals that the drainage vein connects directly to the inferior vena cava in GV without GRS, BRTO may be an effective treatment for GV with GRS.

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Key words: Gastric fundal varices; Gastro-renal shunt; Balloon-occluded retrograde transvenous obliteration

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INTRODUCTION

Rupture of esophagogastric varices is one of the most severe complications of portal hypertension in patients with liver cirrhosis. Gastric varices bleed less frequently than esophageal varices, but bleeding from gastric fundal varices (GV) tends to be more severe and is associated with a high mortality rate[1,2]. Effective management of such bleeding is essential. Endoscopic injection sclerotherapy and endoscopic variceal ligation are currently the mainstay of treatment for esophageal varices. Most cases of GV cannot be treated effectively with endoscopic injection sclerotherapy alone[3-5] because of the rapid blood flow within their vessels with a large diameter. Endoscopic injection of n-butyl-2-cyanoacrylate or isobutyl-2-cyanoacrylate is used as the standard first-line treatment for bleeding GV[6] in the Western countries. However, the reported severe complications related to embolization of other organs have raised concerns for the safety of injection of tissue adhesive agents[7-11]. Transjugular intrahepatic portosystemic shunt (TIPS) placement, one of the interventional radiological techniques, is currently the second-line treatment for bleeding esophagogastric varices[6,12] and can effectively control GV bleeding[13-15]. However, it was reported that this treatment is associated with a lower success rate for GV than for esophageal varices[16-18].

Recently, some newer interventional radiological techniques have been used to treat GV with good results. Balloon-occluded retrograde transvenous obliteration (BRTO) is a very useful treatment for GV in terms of efficacy, safety, and degree of invasiveness[19-29], and the recurrence rate of GV has been also reported to be 0%-10%[23-29] after BRTO, which shows excellent long-term results. Prophylactic treatment with BRTO can effectively prevent GV rupture, and improve patient survival[28]. Ninoi *et al*[29] showed that GV bleeding is better controlled with BRTO than with TIPS. Therefore, not only radiologists but also hepatologists and gastroenterologists in Japan have considered BRTO the standard first-line treatment for GV in place of endoscopic variceal obturation therapy or TIPS. However, it is generally considered that BRTO cannot be used in the treatment of GV without GRS because BRTO is performed through GRS, which exists in almost 85% of GV[7]. It is very crucial to investigate whether BRTO can be feasible for GV without GRS and the effects of BRTO compared with other methods.

The portal hemodynamics of GV without GRS is not well known, and there is no well-established treatment for the eradication of these GV. This study was to examine the portal hemodynamics of GV without GRS compared with those with GRS, and to retrospectively investigate the effects of BRTO compared with some kinds of treatment on the eradication of these varices.

MATERIALS AND METHODS

Ninety-four liver cirrhosis patients at high-risk of GV were treated in our hospital between February 1996 and June 2002 and enrolled in this study. All patients had GV with acute bleeding or were at high-risk of GV. GV were evaluated based on esophagogastroduodenoscopy (EGD) criteria according to the system adopted in Japan[30,31]. In brief, the location of gastric varices (Lg) was classified as being adjacent to the cardiac ring (Lg-c), separated from the cardiac ring (Lg-f), or continuing from the Lg-f to the gastric fundus (Lg-cf). Varices were further classified as straight and small caliber varices (F1), beaded varices (F2), or tumor-shaped varices (F3). We categorized GV as a high risk according to the criteria reported by Kim *et al*[32], who found that a diameter of at least 5 mm and the presence of red spots are independent factors that predict a high-risk of variceal bleeding. All patients were evaluated for variceal hemodynamics with color Doppler endoscopic ultrasonography (CD-EUS) and multidimensional computed tomography (MDCT) or CD-EUS and magnetic resonance angiography (MRA). Of the 94 patients, 23 were urgent or elective cases with episodes of variceal bleeding, and 71 were prophylactic cases. We retrospectively examined their characteristics, liver function, and portal hemodynamics of GV. CD-EUS (FG-32UA, Pentax Co., Ltd., EUB-555 US scanner, Hitachi Co., Ltd., Tokyo, Japan) was performed in all patients to measure the diameters of variceal vessels as well as blood flow velocity before treatment. Blood flow was calculated from blood vessel diameter and blood flow velocity. All patients underwent MDCT or MRA to detect the supply and drainage vein and to determine whether they had a gastro-renal shunt. The patients underwent repeated CD-EUS within 1 wk after treatment to assess the sclerosing effect. The efficiency of each treatment was confirmed by the rate of disappearance of varices. EGD was done every 3-6 mo after treatment to evaluate the status of GV. The recurrence of GV was defined as detection of appearance of high-risk signs (as described above) or variceal bleeding. The follow-up period for recurrence was calculated as days from the date of treatment until the first date when EGD revealed recurrence.

Regarding the selection of the method for treatment, BRTO is the first-line treatment for GV with GRS in our hospital because it is generally considered the first-line treatment in Japan[19-29]. Therefore, in cases where it was possible, we performed BRTO even for varices without GRS. If it was not technically possible to perform BRTO, endoscopic injection sclerotherapy using α-cyanoacrylate glue (CA) or percutaneous transhepatic obliteration (PTO) was used.

BRTO procedure

BRTO is a method for treating GV by injection of a sclerosant after the main drainage vein of GV is blocked angiographically to stagnate blood flow. In general, a balloon catheter is inserted *via* the right femoral vein and wedged into the left adrenal vein. After the balloon is inflated, the gastro-renal shunt is visualized with the contrast agent iopamidol to judge whether shunt occlusion has been achieved. In case of GV without GRS, if the drainage vein is connected directly to the inferior vena cava, BRTO could be performed after an occlusive balloon catheter is placed in the drainage vein through the right femoral vein (Figure 1A and B). If retention of contrast agent is insufficient in GV because of other collateral veins, metallic coils are placed in the collateral veins to reduce the blood flow. In our study, after venography showed sufficient retention of contrast agent in GV, 5% solution of ethanolamine oleate with iopamidol (EOI) was continuously injected through the catheter in the drainage vein until the varices were sufficiently filled with the sclerosant. Thereafter, the catheter was left in place for 24 h to allow sclerosis to occur within the gastric fundal varices.

Modified PTO procedure with injection of sclerosant

The modified PTO using sclerosant and metallic coils[29,33] can embolize GV more selectively than original PTO[34]. In our study, percutaneous transhepatic portography was performed, and the supply and drainage veins of the GV were identified on portography. After metallic coils were placed in the supply vein to reduce blood flow in the GV, EOI was injected through the catheter until the gastric fundal varices were sufficiently filled with the sclerosant.

CA procedure

CA is a method for treating GV by injection of a sclerosant endoscopically. In our study, a sclerotherapy injector, with a 20 gauge needle, was used for variceal injection. The GV were endoscopically punctured and

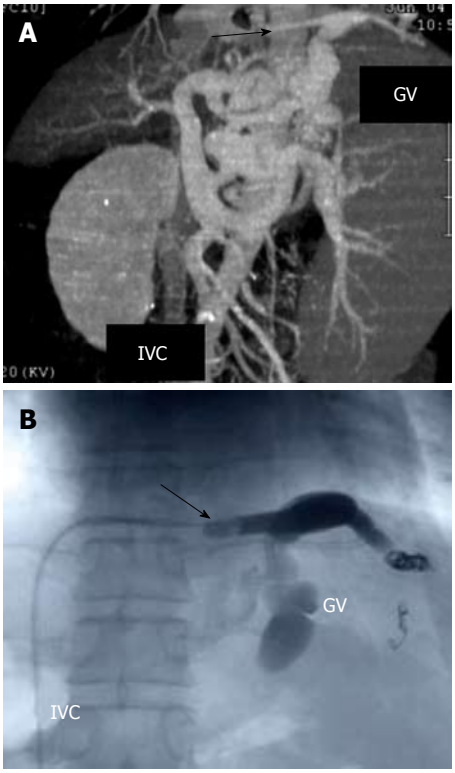


Figure 1 Multidimensional computed tomography (MDCT) revealing the varices supplied by the left gastric vein and drained into the subphrenic vein (arrow) which is connected to the inferior vena cava (IVC) (**A**), and fluoroscopic image of BRTO showing placement of the occlusive balloon catheter in the subphrenic vein (arrow) (**B**). After the blood vessels from the GV except for the main drainage vein were blocked with coils, 5% solution of ethanolamine oleate with iopamidol (EOI) was injected into the varices.

injected with 2.5 mL of a lipiodol-α-cyanoacrylate mixture (64% α-cyanoacrylate) in one shot, and injection was stopped when the varices were filled sufficiently after several injections.

Statistical analysis

Values are presented as mean ± SD, or as percentages. The Mann-Whitney *U* test was used to assess differences in age, sex, form, location, diameter of variceal vessels, blood flow velocity, blood flow volume, and liver function tests. Categorical variables were analyzed by the chi square test, with Yates' correction for continuity where appropriate, or by the Fisher's exact test. The Kaplan-Meier method was used to calculate the recurrence rate for GV. Differences between the groups were compared by means of the log-rank test. Statistical software (SPSS 10.0J, SPSS Japan Inc., Tokyo, Japan) was used in statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Ninety-four patients (60 men, 34 women; mean age 62.3 ± 9.7 years, range 33-80 years) underwent hemodynamic evaluation of GV with CD-EUS and MDCT or CD-EUS and MRA. Among the 94 patients, hepatic function was classified as class A in 53, class B in 36 and class C in 5 by the Child-Pugh classification. The location of GV was at Lg-f in 16 patients and at Lg-cf in 78 patients. The form of gastric varices was F2 in 32 patients and F3 in 62 patients. There were 14 patients who had red color spots on the GV. Among the 94 patients, a GRS was present in 79 (84.0%), and absent in the remaining 15 (16.0%) (Table 1).

Table 1 Clinical characteristics of patients and profile of varices in GRS (+) and GRS (-) groups *n* (%)

	GRS (+) (*n* = 79)	GRS (-) (*n* = 15)	*P* value
Age: yr (range)	61.9 ± 10 (42-80)	61.5 ± 10 (44-76)	NS
Gender (Male:Female)	1:0.58	1:0.5	NS
Child-Pugh classification			
A	47 (59.5)	6 (40)	NS
B	28 (35.4)	8 (53.3)	NS
C	4 (5.1)	1 (6.7)	NS
Endoscopic featres of GV			
Form			
F2	27 (34.1)	5 (33.3)	NS
F3	52 (65.8)	10 (66.7)	NS
Location			
Lgf	12 (15.2)	4 (26.7)	NS
Lgcf	67 (84.8)	11 (73.3)	NS
Presence of red color spots	12 (15.2)	2 (13.3)	NS
History of GV bleeding	20 (25.3)	3 (20)	NS
Finding of CD-EUS			
Blood vessel diameter (mm)	9.5 ± 3.4	6.7 ± 2.8[a]	0.009
Blood flow velocity (cm/s)	16.5 ± 4.3	14.5 ± 3.5	NS
Blood flow volume (cm^3/s)	50.8 ± 45.0	26.1 ± 25.7[a]	0.043

NS: Not significant. GV: Gastric fundal varices; GRS: Gastro-renal shunt; CD-EUS: Color doppler endoscopic ultrasonography. Values for age and findings of CD- EUS are the median. [a]$P < 0.05$ *vs* GRS (+) group.

Regarding the hemodynamics of GV without GRS, there were a number of different supply veins, including the left gastric vein (21.7%), the short gastric vein (18.8%), and the retrogastric vein (59.4%). However, among the 15 cases without GRS, the subphrenic vein was connected to the inferior vena cava as the drainage vein in 13 cases (86.7%) out of the 15 cases without GRS (Table 2). Between the GRS (+) and GRS (-) groups, there were no significant differences in age, sex, form and location of the varices, presence of red-colored spots, velocity of blood flow in the GV, or in Child-Pugh classification. The diameter of varices and blood flow volume were higher in the GRS (+) group than in the GRS (-) group ($P = 0.009$, $P = 0.043$) (Table 1). Regarding the treatment of GV without GRS, we performed BRTO in 6 patients, CA in 4 patients and PTO in 5 patients, without severe complications (Table 2). The GV eradication rate in the GRS (-) group for each procedure was 100%.

The rate of early recurrence, within 6 mo, was 16.7% for BRTO, 50.0% for CA and 40.0% for PTO, respectively, in the GRS (-) group (Table 3). When the main drainage vein was found to be connected to the inferior vena cava, BRTO was the most effective treatment even for GV without GRS. No severe complication occurred in each method except for slight abdominal discomfort or hematuria. Most of the patients treated with BRTO or PTO revealed hematuria caused by sclerosant, but it was recovered when haptoglobin was used without renal damage (haptoglobin was used prophylactically in all cases treated with BRTO or PTO).

Table 2 Gastric fundal varices without gastro-renal shunt

Age	Sex	Form	Diameter (mm)	Verocity (cm/s)	Supplying vein	Drainage vein	Treatment
75	M	LgcfF2	6.8	9.3	Rgv, Sgv	Spv	CA
45	M	LgcF2	8.8	15.0	Lgv, Rgv	Spv	CA
69	F	LgcfF3	12.0	20.1	Lgv	Pev	CA
52	M	LgcfF2	6.1	14.3	Lgv, Sgv	Spv	CA
67	M	LgfF3	4.5	15.0	Lgv, Rgv	Spv, Pcv	PTO
66	M	LgcfF3	4.7	18.5	Lgv, Rgv, Sgv	Pcv	PTO
64	M	LgcF3	4.8	18.1	Rgv	Spv	PTO
65	M	LgcfF3	7.2	10.5	Rgv	Spv	PTO
76	F	LgcF2	3.8	12.0	Lgv	Spv, Pev	PTO
44	F	LgcfF3	8.0	13.0	Rgv, Lgv	Spv, Pcv	BRTO
66	F	LgfF3	11.0	17.7	Rgv	Spv	BRTO
54	M	LgfF3	10.0	17.7	Lgv	Spv	BRTO
52	M	LgfF3	6.0	15.6	Lgv, Rgv, Sgv	Spv, Pcv	BRTO
64	M	LgcfF3	4.4	12.4	Sgv	Spv	BRTO
63	M	LgcF2	2.0	8.9	Lgv	Spv	BRTO

CA: Endoscopic injection sclerotherapy using α-cyanoacrylate glue; Rgv: Retro gastric vein; Sgv: Short gastric vein; Lgv: Left gastric vein; Spv: Subphrenic vein; Pev: Para esophageal vein; Pcv: Peri-cardial vein; BRTO: Balloon occluded retrograde transvenous obliteration; PTO: Percutaneous transhepatic obliteration.

Table 3 Treatment methods and recurrence rate of varices in GRS (-) group *n* (%)

GRS (-)	*n* = 15	*P* value
Treatment		
BRTO	6 (40)	NS
PTO	5 (33)	NS
CA	4 (27)	NS
Early recurrence rate within 6 mo		
BRTO	1 (16.7)	
PTO	2 (40)	
CA	2 (50)	

BRTO: Balloon-occluded retrograde transvenous obliteration; PTO: Percutaneous transhepatic obliteration; CA: Endoscopic injection sclerotherapy using α-cyanoacrylate glue; GRS: Gastro-renal shunt.

DISCUSSION

The present study is the first clinical study examining the portal hemodynamics of GV without GRS compared with those with GRS and the effects of some kinds of treatment for high-risk GV without GRS. The hemodynamic patterns in patients with GV are distinctly different from those in patients with esophageal varices[6,35]. Solitary GV are more frequently supplied by the short and posterior gastric veins, and drain to the inferior vena cava through GRS. However, this shunt is absent in around 15% of patients with GV[25]. The present study revealed that in 13 (87%) of 15 cases without GRS, the subphrenic vein was connected to the inferior vena cava as a drainage vein. Although the varices in the GRS (-) group had thinner veins and a lower blood-flow volume than those in the GRS (+) group, the hemodynamics in the GRS (-) group was more complicated than that in the GRS (+) group, suggesting that it is necessary to examine hemodynamics before treatment, so that the most suitable treatment can be selected.

There is no established treatment for the eradication of GV in these patients without GRS. Our results show that the effect of BRTO on GV without GRS was excellent. The eradication rate of GV without GRS for each procedure (BRTO, PTO, and CA) was high, but BRTO was superior to CA and PTO in early recurrence rate within 6 mo (16.7% for BRTO, 50.0% for CA and 40.0% for PTO). In this respect, no randomized controlled trials comparing BRTO with CA or PTO are available. Regarding the long-term efficacy of CA, GV rebleeding occurs in 23%-50% of patients with most of them occurring in the first year[6]. In our hospital, the recurrence rates at 1, 3, and 5 years of GV with GRS are 2.7%, 2.7%, and 2.7%, respectively, for BRTO in 78 patients[27], 4%, 41.5%, and 53.2%, respectively, for CA in 38 patients, and 28.6%, 42.9%, and 61.9%, respectively, for PTO in 13 patients (data not shown). These results suggest that recurrence rates of GV after BRTO are lower than those of GV after other procedures, showing the excellent long-term effect of BRTO. We speculate that the reason why CA and PTO are associated with a higher recurrence rate than BRTO may be due to insufficient injection of the occlusive substances into the gastric varices and the drainage vein with the PTO and CA procedures, leading to recanalization of the GV. The results of this study suggest that BRTO is an effective treatment for GV without GRS as well as for GV with GRS.

When examination of the hemodynamics reveals that the main drainage vein is connected directly to the inferior vena cava, BRTO is indicated for GV not only with GRS but also without GRS if a catheter can be inserted into the main drainage vein. The purpose of this method is to inject the sclerosant from the drainage vein, so the efficacy of treatment may depend on the sufficient retention of the sclerosant rather than on the approach route. Therefore there is no difference in efficacy depending on the presence or absence of GRS. The most important factor for performing BRTO is the presence of the major shunt directly connected to the inferior vena cava. If the drainage vein is too thin to insert the catheter or not connected to the inferior vena cava, it is impossible to perform BRTO.

Various techniques are now available for the treatment of GV (Figure 2). PTO is used for the treatment of ruptured varices, but the use of this method has decreased following the development of endoscopic treatment. The modified PTO using sclerosant and metallic coils[29,33] can embolize GV more selectively than the original PTO[34]. When the drainage vein is not connected to the inferior vena cava, the modified PTO might be a good option for the treatment of GV without GRS, and some good results have been reported[29,33]. However, this method is not used outside Japan as BRTO. TIPS placement is currently the second-line treatment for bleeding esophagogastric varices[6,12] and can effectively control GV bleeding[13-15]. However, it was reported that its rate (25%-63%) is lower for GV than for esophageal varices[14-16]. Because patients with GV are reported to have a lower portocaval pressure gradient[35], they have extensive spontaneous portosystemic shunts and respond poorly to TIPS. Ninoi *et al*[29] reported that transcatheter sclerotherapy using BRTO

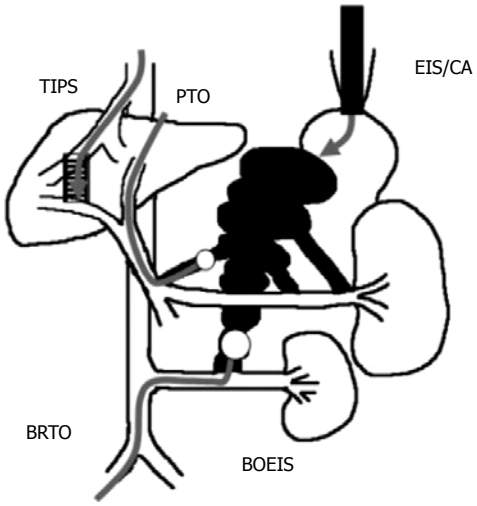


Figure 2 Treatment of gastric fundal varices with balloon-occluded retrograde transvenous obliteration (BRTO), percutaneous transhepatic obliteration (PTO), balloon-occluded endoscopic injection sclerotherapy (BO-EIS), endoscopic injection sclerotherapy using α-cyanoacryrate glue (CA), transjugular intrahepatic portosystemic shunt (TIPS).

and modified PTO can control GV bleeding better than TIPS[29]. However, larger prospective studies need to be performed. We reported that balloon-occluded endoscopic injection sclerotherapy (BOEIS) has an advantage in that it appears to be applicable to patients with GV without GRS[36]. However, BOEIS needs both interventional and endoscopic methods, and is more invasive than other methods. BOEIS is limited only to cases for which other methods are not suitable.

In conclusion, we should examine the hemodynamics before treatment of GV irrespective of the existence of GRS. If this hemodynamic examination reveals that the drainage vein is connected directly to the inferior vena cava in GV without GRS, BRTO might be an effective treatment for GV with GRS as well.

COMMENTS

Background

Recently, a newer interventional radiological technique has been used to treat gastric fundal varices (GV) with good results. Balloon-occluded retrograde transvenous obliteration (BRTO) is a very useful treatment for GV in terms of efficacy, safety, and degree of invasiveness.

Research frontiers

The present study is the first clinical study examining the portal hemodynamics of GV without gastro-renal shunt (GRS) compared to those with GRS and the effects of some kinds of treatment for high-risk GV without GRS.

Innovations and breakthroughs

BRTO is a very useful treatment for GV. However, it is generally considered that BRTO cannot be used in the treatment of GV without GRS. The results of this study suggest that BRTO may be an effective treatment for GV without GRS as well as for GV with GRS.

Applications

When hemodynamic examination reveals that the drainage vein is connected directly to the inferior vena cava in GV without GRS, BRTO might be an effective treatment for GV with GRS as well.

Terminology

BRTO: A method for treating GV by injecting a sclerosant after the main draining vein of GV is blocked angiographically to stagnate for blood flow.

Peer review

In this study, the authors described the hemodynamics and response to different treatments modalities for gastric varices without GRS. The authors support the use of BTRO as the first line treatment for patients in which the drainage vein is connected directly to the inferior vena cava.

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CLINICAL RESEARCH

Effectiveness and safety of herbal medicines in the treatment of irritable bowel syndrome: A systematic review

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Abstract

AIM: To explore the efficacy and safety of herbal medicines (HM) in the treatment of irritable bowel syndrome (IBS).

METHODS: A computer-based as well as manual literature search was performed. We reviewed randomized controlled trials on the treatment of IBS with and without HM.

RESULTS: A total of 22 studies with 25 HMs met the inclusion criteria. Four of these studies were of good quality, while the remaining 18 studies involving 17 Chinese herbal medicine (CHM) formulas were of poor quality. Eight of these reports using 9 HMs showed global improvement of IBS symptoms, 4 studies with 3 HMs were efficacious in diarrhea-predominant IBS, and 2 studies with 2 HMs showed improvement in constipation-predominant IBS. Out of a total of 1279 patients, 15 adverse events in 47 subjects were reported with HM. No serious adverse events or abnormal laboratory tests were observed. The incidence of the adverse events was low (2.97%; 95% CI: 2.04%-3.90%).

CONCLUSION: Herbal medicines have therapeutic benefit in IBS, and adverse events are seldom reported in literature. Nevertheless, herbal medicines should be used with caution. It is necessary to conduct rigorous, well-designed clinical trials to evaluate their effectiveness and safety in the treatment of IBS.

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Key words: Irritable bowel syndrome; Herbal medicine; Systematic review

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INTRODUCTION

Irritable bowel syndrome (IBS) is associated with a variable combination of chronic or recurrent symptoms such as abdominal pain, bloating, constipation and diarrhea. There is generally no structural or biochemical abnormality detected by conventional laboratory tests. IBS is one of the most common functional gastrointestinal disorders accounting for 3% of all primary consultations[1]. In western countries, the prevalence of IBS is around 10%, depending upon the definition used[2]. Moreover, there is increasing prevalence of IBS in the newly developed Asian countries[3]. The potential etiological factors include stress, anxiety, visceral hypersensitivity, altered bowel motility, neurotransmitter imbalances, and inflammation.

Herbal medicines have been used in Asia for a long time. An increasing number of IBS patients are beginning to receive complementary and alternative medicines in the West, most frequently herbal remedies (43%)[4]. Patients may seek HM for symptomatic relief when conventional medicines (CM) are unsuccessful. In such situations, an important question is whether herbal medicines are effective and safe for IBS patients. In the present study, we systematically reviewed the literature and evaluated the effects of HM as well as their potential adverse events in patients with irritable bowel syndrome.

MATERIALS AND METHODS

Search strategy

We carried out a literature search using MEDLINE (1966-2005), EMBASE (1980-2005), Cochrane Database (1992-2005), TCMLARS Database (1984-2005), CJA Full-Text Database (1994-2005), and Chongqing VIP Database (1989-2005) for relevant randomized controlled clinical trials, meta-analysis and systematic reviews published in all languages until October 2005. We used MeSH terms including 'irritable bowel syndrome, functional colonic

disease, drugs, Chinese medicines, traditional medicines, herbal medicines, alternative medicines, complement medicines, plant, oriental traditional medicine' for the database search.

In addition, a hand search of reference lists, review articles, editorials and abstracts from major meetings was also conducted to supplement the electronic search. We included articles published in all languages. Titles and abstracts of all potentially relevant studies were screened before retrieval of the full articles. However, if the title and the abstract were ambiguous, the full articles were scrutinized. Two independent reviewers (J.S and H-X. L) participated in the literature search. Any disagreements were resolved by discussions in order to reach a consensus.

Inclusion and exclusion criteria

The following inclusion criteria were used: (1) Diagnostic criteria used for IBS were ROME I [5] or ROME Ⅱ [6] criteria or the 1986 National symposium on chronic diarrhea (Chengdu China) criteria[7]; (2) Study design with randomized controlled clinical trials, irrespective of blinding; (3) Studies using HM alone for treating IBS in the treatment groups; (4) Identification and description of adverse events; (5) The treatment group received orally administered HM; (5) The control groups received placebo, CM or no treatment.

We excluded studies in which HM was used in combination with CMs, in children and in control groups. Administration of HM by other routes such as injections were also excluded.

Information on HM products derived from a single herb, Chinese proprietary medicines, complex extracts of different herb preparations such as decoction, tablet, capsule, pill, powder and plaster were collected. Standardized extracts of whole plants were included, but isolated 'active' phytochemical ingredients were excluded as these are generally considered as plant chemical products.

Data extraction

Data was collected independently by the reviewers. Any disagreement between the reviewers was resolved by discussion in order to achieve consensus.

Assessment of study quality

The reviewers also assessed independently the quality of each study by Jadad scale[8] and Cochrane Handbook[9].

Statistical analysis

The relative risk (RR) and 95% confidence interval (95% CI) were calculated using raw data derived from each study. Intention-to-treat analysis was performed if possible. Meta-analyses was performed with either fixed effects model or random effects model according to the presence or absence of heterogeneity when HM was compared with control. Statistical analysis was performed with RevMan 4.2 to detect any bias in studies using the funnel plot.

RESULTS

Our initial search generated 572 citations. After analyzing the titles and abstracts, and reading the full text articles, only 22 studies[10-31] with 25 arms involving 1279 patients and 763 controls met the predefined inclusion criteria. Eighteen of the studies were conducted in China and published in Chinese, four were published in English, one each from Australia[11] and Israel[20], and two from Germany[29,31] (Table 1).

Three studies[11,20,29] used computer software and two[21,25] used random number tables to generate the allocation sequence. Four studies[11,20,29,31] used an adequate concealed allocation method of randomization. Six studies[11-12,20,27,29,31] used placebo as control, four of these studies[11,20,29,31] were considered to be adequate, while two[12,27] demonstrated an inadequate comparison between placebo and CTM decoction; Four studies[11,20,29,31] provided statistical data with intention-to-treat protocol.

Using the Jadad Score and Cochrane handbook, four studies[11,20,29,31] were judged to be of high quality, whereas the remaining reports were of poor quality.

Efficacy of herbal medicines

Global symptoms of IBS: Two studies[11,29] with 3 HMs showed significant benefit compared to placebo with respect to the global improvement of IBS symptoms: standard CHM formula[11] (RR 2.15; 95% CI: 1.26-3.65 rated by patient and RR 2.62; 95% CI: 1.44-4.78 assessed by gastroenterologist), STW5[29] (RR 1.68; 95% CI: 1.13-2.51), STW5-Ⅱ[29] (RR 1.90; 95% CI: 1.30-2.78) (Figure 1). The following seven CHMs using complex herbal formulas appeared to be effective in improving global IBS symptoms compared to CM: Lizhong huoxie decoction[10] (RR 1.40; 95% CI: 1.11-1.76), Huatan Liqi Tiaofu decoction[13] (RR 1.24; 95% CI: 1.05-1.47), Geqin Shujiang Saocao decoction[14] (RR 1.24; 95% CI: 1.05-1.47), Huanchang decoction[16] (RR 1.41; 95% CI: 1.08-1.84), Congpi Lunzhi Formula[17], (RR 1.74; 95% CI: 1.35-2.24), Xiangsha Liujunzi decoction[18] (RR 1.28; 95% CI: 1.00-1.63, $P = 0.015$), and Shunji mixture[19] (RR 1.23; 95% CI: 1.01-1.49).

By contrast, the following compounds were not effective in the treatment of global IBS symptoms compared to placebo or CM: Individualized CHM[11] (RR 1.51; 95% CI: 0.83-2.73 assessed by patients and RR 1.54; 95% CI: 0.77-3.05 assessed by gastroenterologist), Bitter candytuft[29] (RR 1.23; 95% CI: 0.78-1.92), Curcuma [31] (RR 0.97; 95% CI: 0.60-1.55), Fumitory[31] (RR 1.13; 95% CI: 0.74-1.72) (Figure 1), Changkang Capsule[21] (RR 1.03; 95% CI: 0.92-1.14), and Jiejing Yiji decoction[30] (RR 1.09; 95% CI: 0.96-1.23) (Figure 2).

Diarrhea: As shown in Figure 3, a meta-analysis of Tongxie Yaofang modified decoction[12] and Tongxie Yaofang plus Sini San decoction[27] (RR 1.14; 95% CI: 1.04-1.24) showed that compared to CM these products had antidiarrheal effects in patients with diarrhea-predominant IBS patients. We combined the data from these two studies[12,27] since their herbal ingredients and dosages were very similar. Their effects were similar to Liyiting decoction[26] (RR 1.28; 95% CI: 1.02-1.62), and Tongxie yihao capsule[28] (RR 1.22; 95% CI: 1.01-1.46).

Three studies demonstrated an insignificant improvement in diarrhea: Xianshi Capsule[15] (RR 1.20; 95% CI: 0.98-1.48), Changning Yin decoction[24] (RR 1.19; 95%

Table 1 Characteristics of studies included in the analysis

Study ID	Year	*n*	Mean age	Sex (male %)	Study quality	Herbal medicine	Type of herbs	Control	Length (wk)	Follow-up (wk)
Gao[10]	1992	111	34	39	L	Lizhong Huoxie decoction	C.F	Oryzanol, Nifedipine	4	52
Bensoussan[11]	1998	116	47	35	H	Individualized CHM Standard Formula	C.F C.F	Placebo	16	14
Zhao[12]	2000	233	39	34	L	Tongxie Yaofang modified decoction	C.F	Salazosulfapyridine Diphenoxylate, Anisodamine, Amitriptyline, Placebo	3	n.r
Lei[13]	2000	96	39	56	L	Huatan Liqi Tiaofu decoction	C.F	Smecta	3	n.r
Wang[14]	2000	96	39	49	L	Geqin Shujiang Shaocao decoction	C.F	Smecta, vitB	3	n.r
Ye[15]	2002	80	37	46	L	Xianshi Capsule	C.F	Dicetel, Smecta	8	n.r
Deng[16]	2002	62	38	35	L	Huanchang decoction	C.F	Anisodamine, Oryzanol	3-6	52
Zeng[17]	2002	98	38	46	L	Congpi Lunzhi Formula	C.F	Bacillus Licheniformis	6	n.r
Ge[18]	2002	57	40	44	L	Xiangsha Liujunzi decoction	C.F	Diazepam, Propantheline, Domperidone	2	n.r
Zhou[19]	2002	105	n.r	n.r	L	Shunji mixture	C.F	Bitinal	4	n.r
Sallon[20]	2002	80	47.9 ± 2.1[1] 46.3 ± 2.9[3]	38	H	Padma Lax (Tibetan herbal formula)	C.E	Placebo	12	n.r
Ma[21]	2003	204	36	48	L	Changkang Capsule	C.F	Amitriptyline	4	n.r
Wang[22]	2003	104	53	53	L	Wuma Simo decoction	C.F	Cisapride	2	n.r
Liu[23]	2003	77	37	36	L	Gegan Qinlian Pellet	C.F	Nifedipine	3	n.r
Li[24]	2003	101	39	68	L	Changning Yin decoction	C.F	Diphenoxylate	5	n.r
Shen[25]	2003	47	41.6 ± 12.8[1] 42.3 ± 14.7[3]	57	L	Changjitai decoction	C.F	Dicetel	8	n.r
Zhao[26]	2004	84	44	44	L	Liyiting decoction	C.F	Dicetel	6	n.r
Xiao[27]	2004	167	37	41	L	Tongxie Yaofang plus sini san decoction (similar with Tongxie Yaofang modified decoction)	C.F	Salazosulfapyridine Diphenoxylate, Anisodamine, Amitriptyline, Placebo	2	n.r
Gao[28]	2004	98	36	35	L	Tongxie yihao capsule	C.F	Dicetel, Domperidone, Loperamide, Doxepin	4	24
Madisch[29]	2004	208	47	40	H	1 STW5 2 STW5- Ⅱ 3 Bitter Candytuft	C.E C.E M.E	Placebo	4	n.r
Bo[30]	2004	92	38.8 ± 1.7[1] 41.3 ± 1.7[3]	41	L	Jiejing Yiji decoction	C.F	Cerekinon	2	4
Brinkhaus[31]	2005	106	47.2 ± 11.7[1] 49.5 ± 14.5[2] 49.0 ± 9.1[3]	37	H	1 Curcama 2 Fumitory	M.E M.E	Placebo	18	n.r

H: High quality study; L: Low quality study; C.F: Complex formulation of herbs; C.E: Complex extracts of different herbs; M.E: Mono-extract of single herb. [1]The first treatment group; [2]The second treatment group; [3]The control group; n.r: Not reported.

CI: 0.99-1.42), Changjitai decoction[25] (RR 1.14; 95% CI: 0.81-1.60) (Figure 3).

Constipation: One study[20] showed that compared to placebo Padma Lax was more effective in relieving symptoms in patients with constipation-predominant IBS (RR 7.24; 95% CI: 2.37-22.12) (Figure 4). Similarly, another study[23] demonstrated that Gegan Qinlian pellet was therapeutically effective (RR 1.28; 95% CI: 1.03-1.60). By contrast, Wuma Simo decoction[22] had no advantages over CMs in the patients with constipation-predominant IBS (RR 1.07; 95% CI: 0.91-1.25) (Figure 5).

Therapeutic period and follow-up

The duration of treatment was 8 weeks or more in five studies[11,15,20,25,31], and 5 or 6 wk in three studies[17,24,26]. In one study[16] the treatment duration was 3-6 wk, whereas the other studies[10,12-14,19,21,23,28,29] lasted 3 or 4 wk, while the shortest[18,22,27,30] period of the treatment was only 2 wk. Five studies[10-11,16,28,30] reported follow-up assessment after herbal medicine treatment. Two[10,28] reported that the symptom recurrence rates were lower in the treatment group compared to the conventional treatment group (25.5% *vs* 60%; 23.1% *vs* 50%, $P < 0.01$), after one-year and one-half-year respectively, following completion of the treatment. Another study[11] presented the result as bowel symptom scale. There was significant improvement in the individualized group (75%), and standard group (63%) compared with placebo group (32%) after 14 wk of follow-up. One study[16] reported the number of subjects that were lost to follow-up, but the reasons were not provided. Another study[30] reported symptom recurrence in 3 of 48 patients in the treatment group after 2 wk without treatment.

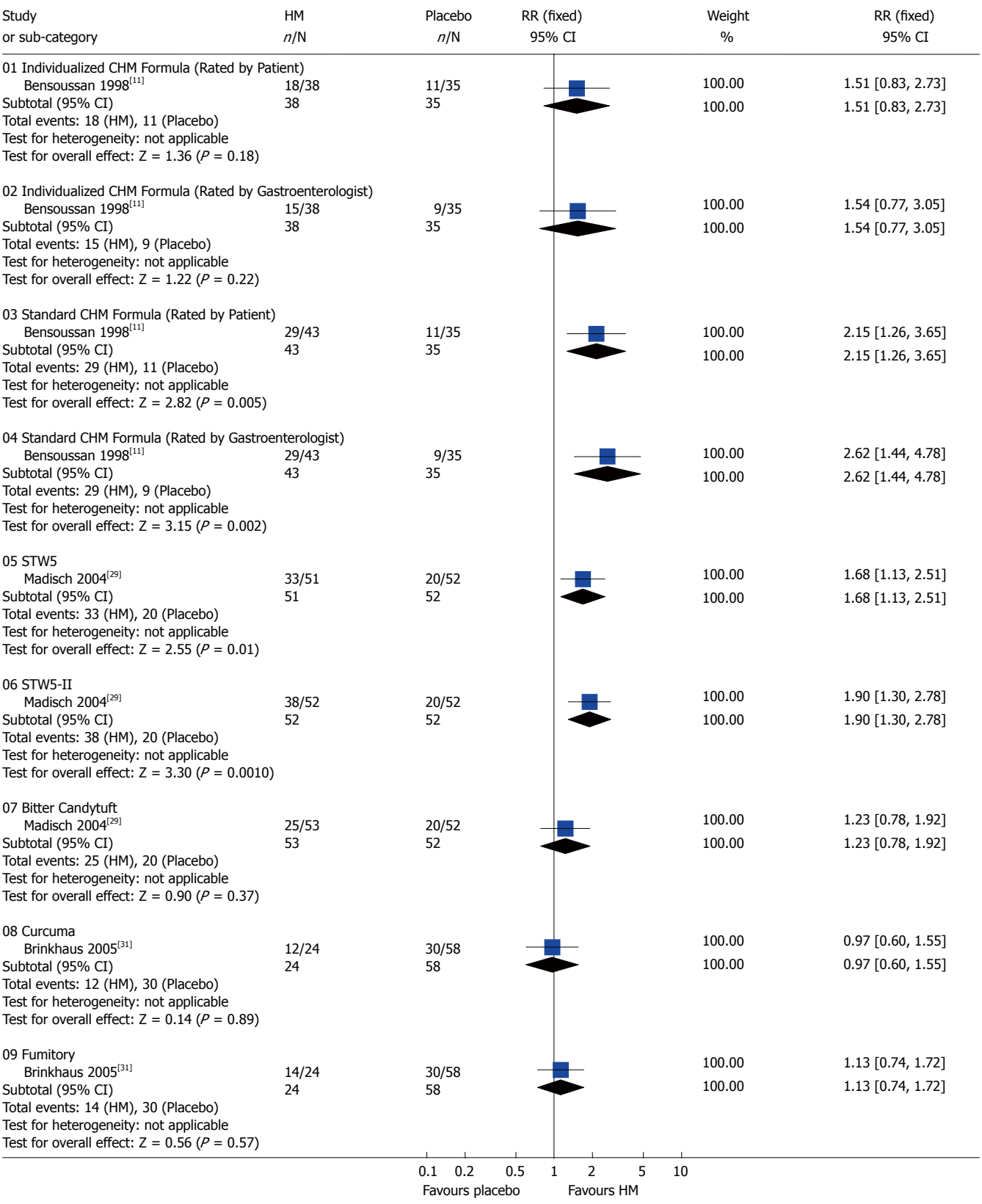


Figure 1 Comparison of herbal medicine and placebo (Outcome: global improvement of symptoms). HM: Herbal medicine; RR: Relative risk; CI: Confidence interval; Fixed: Fixed effects model.

Adverse effects of herbal medicine

In the total study group of 1279 patients, only 15 adverse events in 47 subjects were observed (Table 2). The most common symptoms were abdominal distention, constipation and abdominal pain. None of the subjects developed any serious adverse events or abnormal laboratory tests. The percentage of adverse events associated with HM was 3.67% (95% CI: 2.64-4.71%) in the 1279 patients in the different treatment groups.

Bias analysis

Funnel plots indicated an asymmetry (Figure 6).

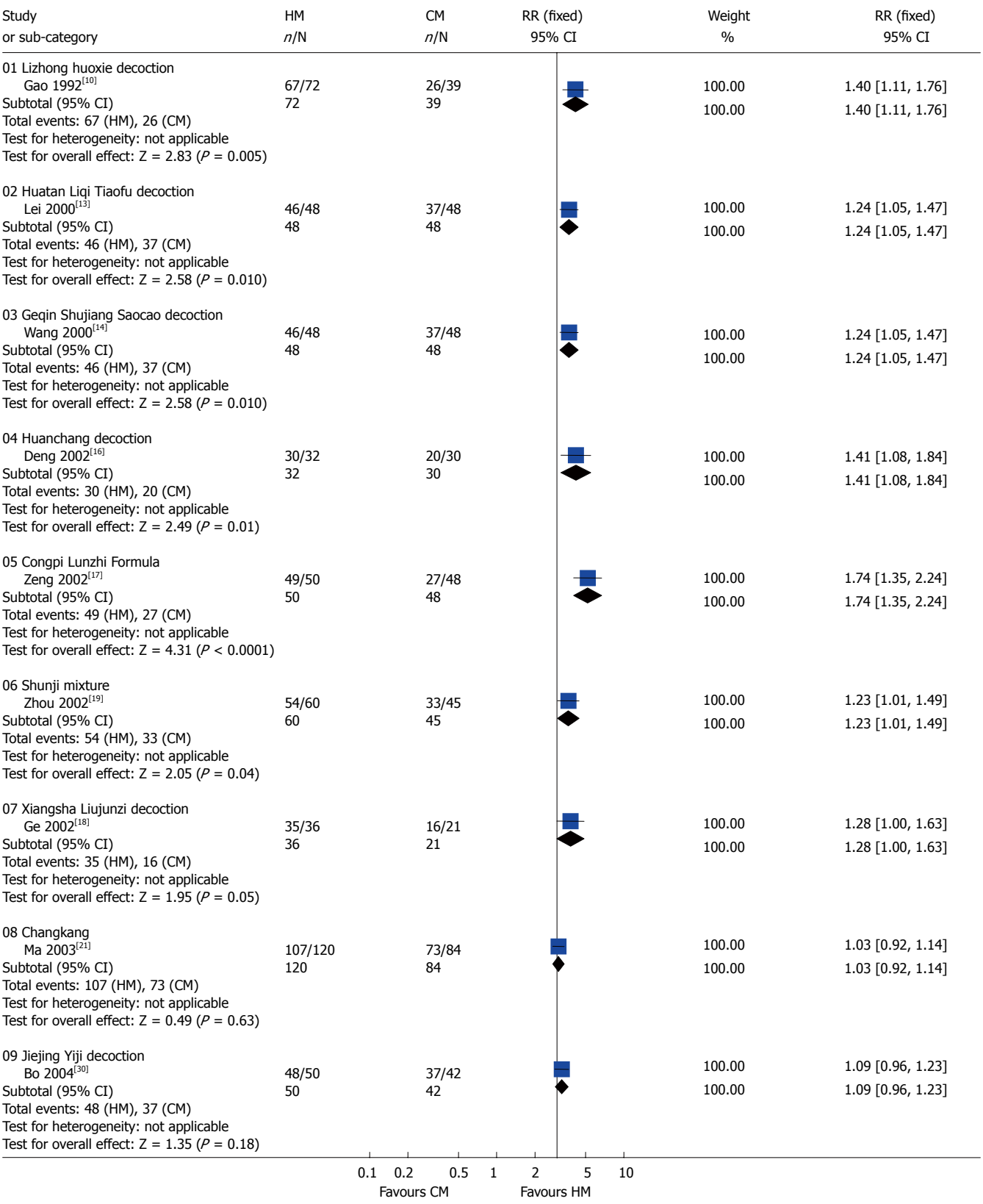


Figure 2 Comparison of herbal medicine and conventional medicine (Outcome: Global improvement of symptoms). HM: Herbal medicine; CM: Conventional medicine; RR: Relative risk; CI: Confidence interval; Fixed: Fixed effects model.

DISCUSSION

In the present review, 3 out of the 4 good quality studies[11,20,31] demonstrated 4 different herbal interventions: one Chinese herbal medicine (standard formula), one Tibetan herbal formula (Padma Lax) and two complex extracts of herbs: STW5 and STW5-Ⅱ which could potentially relieve abdominal pain, constipation, diarrhea and alternating constipation and diarrhea. Moreover, three[11,29,31] out of these four studies showed that four

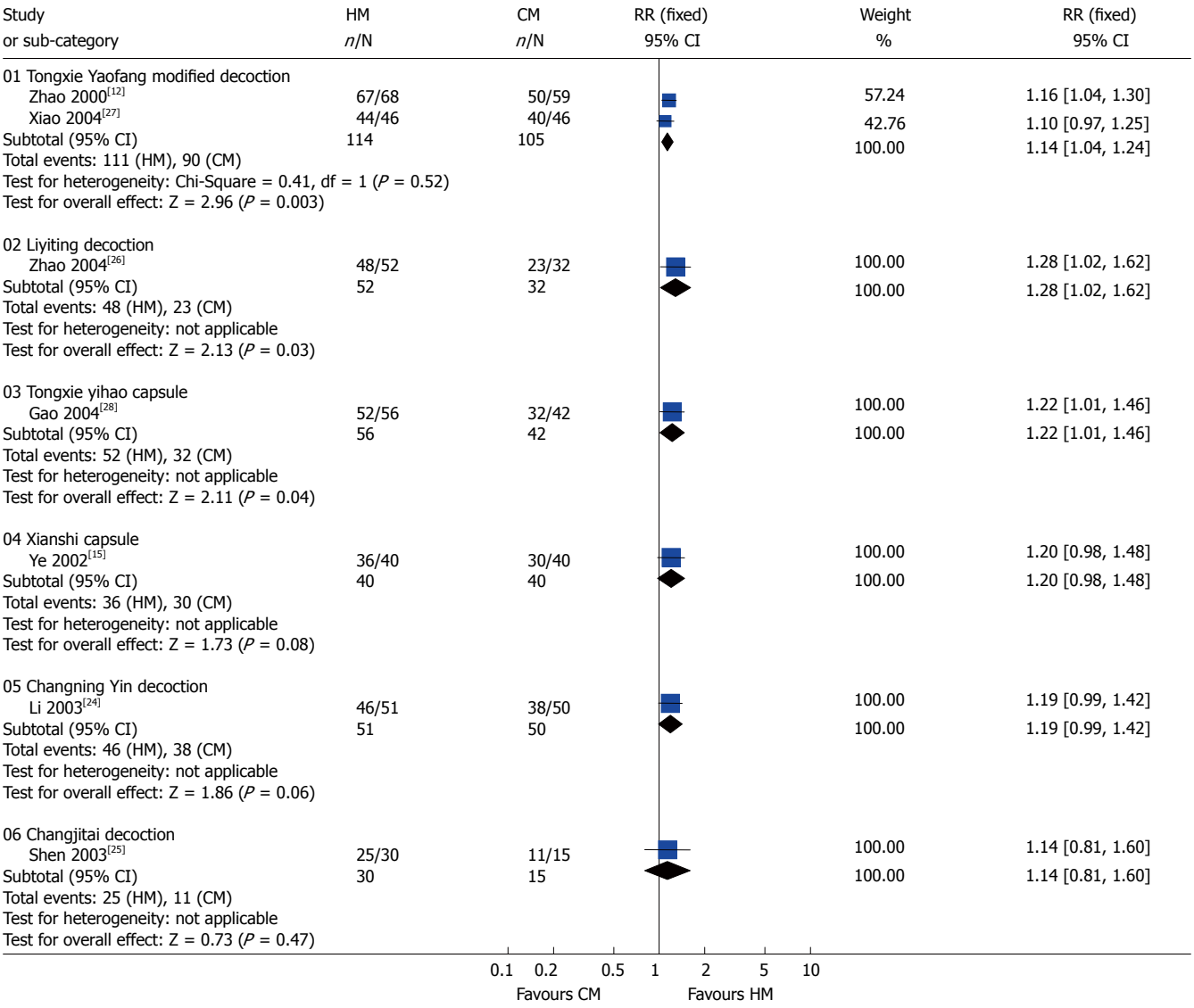


Figure 3 Comparison of herbal medicine and conventional medicine (Outcome: diarrhea). HM: Herbal medicine; CM: Conventional medicine; RR: Relative risk; CI: Confidence interval; Fixed: Fixed effects model.

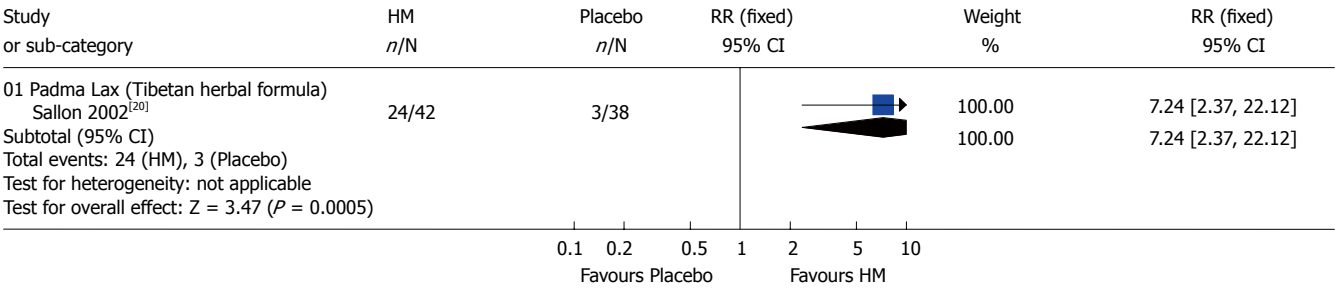


Figure 4 Comparison of herbal medicine and placebo (Outcome: constipation). HM: Herbal medicine; RR: Relative risk; CI: Confidence interval; Fixed: Fixed effects model.

interventions including one individualized formula and three mono-extracts of single herb (Bitter Candytuft, Curcama and Fumitory) were not effective in IBS. We recognized that some complex herbal formulas may improve IBS symptoms, whereas three mono-extracts of single herbs had no beneficial effect. A possible explanation for these findings is that the therapeutic effect may be enhanced by the synergic actions of compounds in a mixture of different herbs.

Twelve[10,12-14,16-19,23,26-28] out of the 18 poor quality studies showed that some Chinese herbs formulas, such as, Huatan Liqi Tiaofu decoction, Tongxie Yaofang modified and Tongxie Yaofang plus Sini San decoction, Geqin Shujiang Saocao decoction, Huanchang decoction, Congpi Lunzhi Formula, Xiangsha Liujunzi decoction, Shunji mixture, Gegan Qinlian Pellet, and Liyiting decoction were more beneficial than CMs in the treatment of IBS.

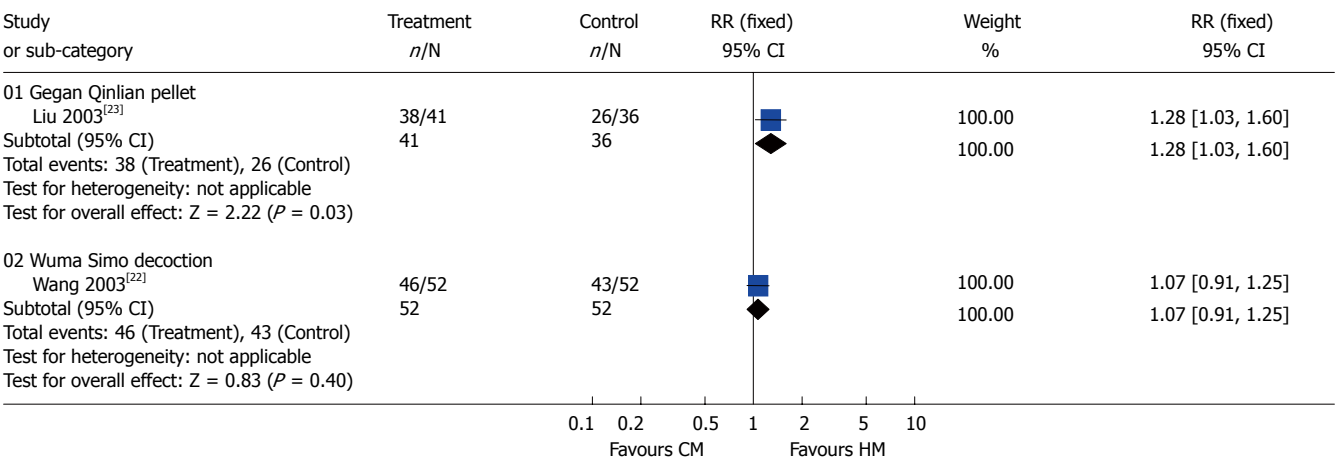


Figure 5 Comparison of herbal medicine and conventional medicine (Outcome: constipation). HM: Herbal medicine; CM: Conventional medicine; RR: Relative risk; CI: Confidence interval; Fixed: Fixed effects model.

Table 2 The type and frequency of adverse events reported in the 22 studies included in the analysis (n = 1279)

Adverse events	Number of adverse events	Percentage	95% CI
Distention	9	0.70	0.32-1.34
Diarrhea	8	0.63	0.27-1.23
Abdominal pain	6	0.47	0.17-1.02
Constipation	5	0.39	0.13-0.91
Dizziness and sleepiness	4	0.31	0.09-0.80
Headaches	4	0.31	0.09-0.80
Nausea	3	0.23	0.05-0.69
Gastrointestinal discomfort	1	0.08	0.002-0.44
Upper gastrointestinal discomfort	1	0.08	0.002-0.44
Loss of hair	1	0.08	0.002-0.44
Pruritus	1	0.08	0.002-0.44
Paraesthesia	1	0.08	0.002-0.44
Disturbance	1	0.08	0.002-0.44
Hoarseness	1	0.08	0.002-0.44
Shortness of breath and chest pain	1	0.08	0.002-0.44
Total	47	3.67	2.73-4.87

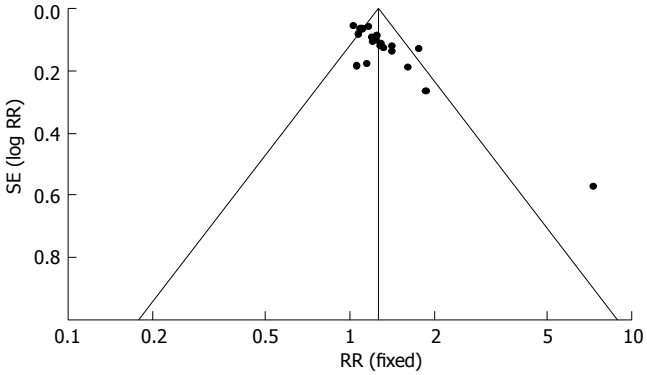


Figure 6 Funnel plot. Fixed: Fixed effects model; RR: Relative risk.

However, these studies revealed several methodological flaws. We found that the longest duration of treatment was 18 wk, while the shortest was just 2 wk, and only 5 studies lasted more than 8 wk. The most frequent treatment duration was 3-4 wk. With such a short period of treatment, it is hard to reach the therapeutic goal in IBS. Some studies reported the long-term effects of herbs and the rate of symptom recurrence. Thus, it is necessary to have a relatively long treatment duration with herbal medicines as well as the duration of the follow-up period; Funnel plot of inclusion trials indicated asymmetry, the major interpretation is the presence of publication bias and variable methodological quality. In the studies that we reviewed, 18 out of 22 were conducted in China and published in Chinese. Chinese studies more frequently showed favorable therapeutic results compared to articles in English, particularly those with a high rate of positive outcome (99%)[32]. The large number of poor quality studies is another source of bias. Furthermore, the small size of studies and the variability of the control treatment may cause asymmetry of the funnel plot. We noticed that most of the studies with Chinese herbs were of poor methodological quality and would not provide strong evidence to confirm the efficacy of CHM. However, the lack of good evidence supporting the effectiveness of CHM does not mean that these preparations are not effective in the treatment of IBS, instead we need to improve the methodological quality of trials in order to verify the efficacy of CHM as a therapeutic approach. We agree with the opinion of Liu[33] that the potential beneficial effects of CHM need to be confirmed in rigorous trials with well-designed, randomized, double blinded, placebo controlled studies. A good example is the study performed by Bensoussan and colleague[11].

There is growing interest in placebo response in patients with IBS. A systematic review of RCTs showed that global improvement in IBS symptoms with placebo was 40.2% (range 16%-71.4%)[34].Other investigators have reported placebo response rate of 57% in IBS[35]. Placebo response rate correlated with factors such as frequency of intervention, methodological quality of study, duration of the study, the patient-practitioner interaction and the diagnosis treated[34,36-38]. Since the number of studies using placebo were small, we did not explore the response effect of placebo in the present study.

In the 22 trials that we reviewed, there were only 15 adverse events associated with HM. These were abdominal distention, constipation, abdominal pain, diarrhea, dizziness and hypersomnia, headache and nausea. However, no serious side effects or abnormalities of laboratory parameters such as liver function, renal function or haematological tests were reported with the treatment. Studies conducted in the West reported more adverse events than those from China. It is possible that because of the lack of rigorous monitoring, several adverse effects including serious events may not have been reported. Similarly, because of publication bias, adverse events related to herbal medicine may not be reported properly.

In summary, the use of HM for treating IBS is increasing worldwide. Most of the studies included in our review showed a beneficial effect on IBS symptoms. However, the methodological quality of the studies was variable, with 82% being of poor quality which may have overestimated the effectiveness of treatment. Although adverse events arising from the use of herbs were mild and infrequent, HM should be used with caution because of the reasons discussed above. It is therefore necessary to conduct Level I studies in order to provide evidence for Grade A recommendations[39] and clarify whether Chinese herbal medicines are reliable and safe therapy in IBS.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common functional bowel disorder that affects the patient's quality of life. No single treatment is reliably effective, and an increasing number of patients worldwide are seeking herbal medicines to cure their illness.

Research frontiers

We assessed the efficacy and adverse events of herbal medicines in IBS.

Innovations and breakthroughs

We made a comprehensive search of studies using herbal medicines for the treatment of IBS. The studies were analyzed to determine if herbal medicines are appropriate for IBS patients.

Applications

Based on this evaluation, we concluded that herbal medicines could not be reliably recommended because of methodological flaws in the studies. Further studies of better methodological quality should be carried out to determine the efficacy of herbal medicines in IBS.

Peer review

The authors explored the efficacy and safety of herbal medicines (HM) in the treatment of IBS. It was concluded that herbal medicines have therapeutic benefit in IBS.

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RAPID COMMUNICATION

Enteral glutamine pretreatment does not decrease plasma endotoxin level induced by ischemia-reperfusion injury in rats

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Abstract

AIM: To investigate whether oral glutamine pretreatment prevents impairment of intestinal mucosal integrity during ischemia-reperfusion (I/R) in rats.

METHODS: The study was performed as two series with 40 rats in each. Each series of animals was divided into four groups. The first group was used as a control. Animals in the second group were only pretreated with oral glutamine, 1 g/kg for 4 d. The third group received a normal diet, and underwent intestinal I/R, while the fourth group was pretreated with oral glutamine in the same way, and underwent intestinal I/R. Intestinal mucosal permeability to ^{51}Cr-labeled EDTA was measured in urine in the first series of animals. In the second series, histopathological changes in intestinal tissue and plasma endotoxin levels were evaluated.

RESULTS: Intestinal I/R produced a significant increase in intestinal permeability, plasma endotoxin level and worsened histopathological alterations. After intestinal I/R, permeability was significantly lower in glutamine-treated rats compared to those which received a normal diet. However, no significant change was observed in plasma endotoxin levels or histopathological findings.

CONCLUSION: Although glutamine pretreatment seems to be protective of intestinal integrity, upon I/R injury, such an effect was not observable in the histopathological changes or plasma endotoxin level.

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Key words: Endotoxin; Glutamine; Intestinal permeability; Ischemia-reperfusion injury

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INTRODUCTION

Intestinal injury as a result of ischemia and subsequent reperfusion plays an important role in a variety of clinical conditions such as shock, and in those undergoing cardiovascular surgery[1]. In these situations, the small intestine may suffer ischemia of varying duration. Intestinal ischemia-reperfusion (I/R) disrupts intestinal mucosal integrity[2,3], and causes an increase in intestinal permeability[4,5] and bacterial translocation[6]. Plasma endotoxin level also increases after I/R[6]. All these have been increasingly recognized as potential causes of multi-system organ failure in critically ill patients[7-9].

Intestinal permeability is assessed non-invasively *in vivo* by measuring urinary excretion of orally administered test substances. Lactulose, various polymers of polyethylene glycol, ^{51}Cr-labeled EDTA and ^{99m}Tc diethylenetriaminepentaacetate are the most commonly used test substances. Test results may be influenced by a change in any of the pre- or post-mucosal factors, apart from intestinal permeability itself[10]. Measurement of intestinal permeability by some test substances may give an idea about some harmful factors such as microorganisms passing through the mucosal barrier, from the intestinal lumen to the systemic circulation. It is thought an increase in plasma endotoxin level is one of the responsible factors which may contribute to the clinical results of intestinal I/R injury[11]. Measurement of plasma endotoxin levels seems to be important in assessing the systemic effect of intestinal I/R.

Glutamine (Gln) is a non-essential amino acid, and the most abundant free amino acid in whole blood and the

intracellular amino acid pool[12]. It is an important respiratory fuel, and nucleotide precursor for the gastrointestinal tract[13]. Gln-supplemented parenteral nutrition protects rats against morphological and functional mucosal injury[14], and improves survival in animals after intestinal I/R[15]. Some other experimental studies have also shown that intraluminal injection of Gln protects the mucosa, and diminishes the accumulation of neutrophils in the lamina propria of the small bowel during I/R[16]. Generation of reactive oxygen intermediates (ROI) during reperfusion is thought to be one of the major causes of intestinal mucosal injury[17,18]. Gln is also essential for the synthesis of the intrinsic ROI scavenger glutathione. Therefore, this protective action may be due to augmentation of ROI scavenging in intestinal mucosa[19].

In this study, we aimed to investigate the effects of orogastric Gln pretreatment on intestinal mucosal permeability, plasma endotoxin level and intestinal histopathology during intestinal I/R injury in rats.

MATERIALS AND METHODS

Experimental design

The experiment was performed in female Wistar albino rats weighing 200-230 g. It was approved by the Ankara University Ethical Committee, and was conducted according to the European Community guidelines for the use of experimental animals. Animals were fed with their ordinary diet and allowed to drink water *ad libitum*. Owing to the time difference between urine collection and blood sampling, the study had to be completed in two series of experiments (Table 1).

Table 1 Characteristics of study groups

Groups	Measurement of intestinal permeability by ^{51}Cr-EDTA		Measurement of plasma endotoxin levels and histopathological changes	
	Gln	I/R	Gln	I/R
Ⅰ (Control)	–	–	–	–
Ⅱ (Gln)	+	–	+	–
Ⅲ (I/R)	–	+	–	+
Ⅳ (I/R and Gln)	+	+	+	+

Time-matched, sham-operated animals undergoing laparotomy and dissection of the SMA without occlusion served as controls (group Ⅰ). The Gln group (group Ⅱ) was pretreated with Gln (1 g/kg per day) by the orogastric route for 4 d[20,21]. Gln was prepared in 0.9% NaCl for daily use. Intestinal I/R group (group Ⅲ) underwent 1 h intestinal ischemia, and 1 h reperfusion. In the I/R and Gln group (group Ⅳ), I/R periods and Gln administration were the same as in Gln (Ⅱ) and I/R (Ⅲ) groups.

Induction of intestinal I/R

A rat model of transient mesenteric occlusion was used to obtain intestinal I/R. Rats were anesthetized with ketamine (80 mg/kg, im) and xylazine (10 mg/kg, im). Intestinal I/R was induced by 60 min occlusion, followed by 60 min reperfusion[22]. During the 2 h of the surgical procedure, animals were kept at room temperature, and given intraperitoneal fluid as 0.9% NaCl (10 mL/kg). The superior mesenteric artery (SMA) was exposed through a midline abdominal incision, and both this artery and the collateral branches coming from the celiac axis, and the inferior mesenteric artery were occluded with atraumatic vascular clamps (Vascu-Statt Ⅱ original No. 1001-532-3; Scanlan International, St Paul, MN, USA) for 1 h, followed by 1 h reperfusion. Existence of pallor and absence of pulsation ensured mesenteric occlusion during the ischemic period. Recovery of pulsation and pink color were controlled in each animal when the clamps were removed. The existence of intestinal I/R in this model was also confirmed in our laboratory by the appearance of pulses at the marginal arteries (direct vision of mesenteric circulation by microscopy), as well as by fluorescein angiography in preliminary experiments[23].

Measurement of intestinal mucosal permeability

Intestinal mucosal permeability was measured on the basis of urinary radioactivity levels following oral administration of ^{51}Cr-EDTA. ^{51}Cr-EDTA was employed as a well accepted marker of mucosal integrity[24,25]. After 60 min reperfusion, rats were given 5 μCi ^{51}Cr EDTA in 0.5 mL saline solution by the orogastric route. Urine samples were collected in metabolic cages for 6 h following the reperfusion period. During urine collection, animals did not receive any food; however, they were allowed to access tap water. The level of radioactivity in the urine samples of 500 μL was then determined by counting on a gamma counter (DPC Gambyt CR, Los Angeles. USA). The amount of ^{51}Cr-EDTA excreted in urine during 6 h was calculated as a percentage of the ingested dose.

Measurement of plasma endotoxin level

Plasma endotoxin level was measured by the colorimetric Limulus amebocyte lysate (LAL) test. The test was performed by using the Pyrochrome test kit (Pyroquant Diagnostik, Mörfelden, Germany). All glassware, solutions and surgical instruments used in the experiment were autoclaved at 121℃ for 15 min. The non-pyrogenicity of solutions was tested using the LAL test (Charles River Endosafe, Charleston, SC, USA).

Venous blood samples (3-4 mL) were collected using heparin-coated pyrogen-free disposable syringes. Platelet-rich plasma (PRP) was prepared from the blood by centrifugation at 150 g for 10 min. Fifty microliters of PRP was transferred into a polystyrene plastic tube and kept frozen at -80℃ until the assay. Frozen PRP samples were kept at room temperature for about 30 min before the assay. Fifty microliters of 0.18 mol/L NaOH was added to 50 μL PRP, and incubated at 37℃ for 5 min. Next, 50 μL 0.32 mol/L perchloric acid was added, and incubated at 37℃ for a further 10 min. To dissolve the formed precipitate, 100 μL 0.18 mol/L NaOH was added, and vortexed. Twenty-five microliters of the solution was transferred into sterile non-pyrogenic microplates (Pyroquant Diagnostik), and 25 μL 0.2 mol/L Tris/HCl buffer (pH 8.0) was added to the wells[26]. Finally, 50 μL pyrochrome test solution was added to all wells, and mixed for 30 s. Plates were incubated at 37℃. Optical density was read at 405 nm. Standard curves from 0.04 to 1.28 EU/mL were used to evaluate the concentration of endotoxin.

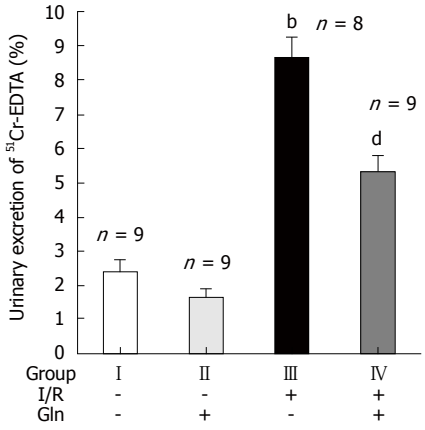


Figure 1 The effect of enteral Gln pretreatment (1 g/kg per day for four days) on intestinal permeability during I/R injury in rats. Intestinal I/R was induced by 60 min of occlusion, followed by 60 min of reperfusion. Changes in intestinal permeability were measured by urinary excretion of ^{51}Cr-EDTA after its oral administration in rats. Data are expressed as means ± SEM. $^{b}P < 0.001$ *vs* group Ⅰ, Ⅱ and Ⅳ; $^{d}P < 0.001$, *vs* groups Ⅰ, Ⅱ and Ⅲ.

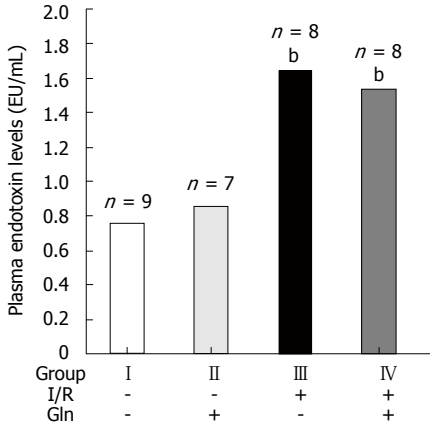


Figure 2 The effect of enteral Gln pretreatment (1 g/kg per day for four days) on plasma endotoxin levels during I/R injury in rats. Intestinal I/R was induced by 60 min of occlusion, followed by 60 min of reperfusion. Changes in plasma endotoxin levels were measured by LAL test. Data are expressed as medians. $^{b}P < 0.001$ *vs* groups Ⅰ and Ⅱ.

Results were calculated by using non-linear regression of a four-parameter logistic model.

Histopathological assessment of ileal tissues

After the collection of blood samples, ileal tissue samples, 10 cm proximal to the cecum, were harvested and evaluated for histopathological changes. Sections were stained with hematoxylin and eosin, and were examined by light microscopy by two pathologists in a blinded manner. Mucosal injury was scored on a scale from 0 to 5, as described by Chiu *et al*[2].

Statistical analysis

The SPSS program was used for statistical analysis. Comparison of the various protocols on the changes in intestinal permeability was made by one-way analysis of variance (ANOVA) following a Bonferroni post-hoc test. Changes in plasma endotoxin levels, and intestinal histopathology were determined using a Kruskal-Wallis test following a multiple comparison post-hoc test[27]. Data on changes in intestinal permeability were presented as means ± SEM. Data of plasma endotoxin levels, and intestinal histopathology were presented as medians. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Gln pretreatment on intestinal permeability during I/R injury

To investigate the effect of intestinal I/R and Gln pretreatment on intestinal permeability, renal clearance of ^{51}Cr-EDTA was assessed. Statistically significant differences were detected in intestinal permeability in the I/R group when compared to the control group (8.6% ± 1.7% *vs* 2.4% ± 1.1%, $P < 0.001$). Gln pretreatment significantly lowered the increased intestinal permeability due to intestinal I/R (8.6% ± 1.7% *vs* 5.3% ± 1.3%, $P < 0.001$). There was no statistically significant difference in intestinal permeability between the control and Gln groups (Figure 1).

Effect of Gln pretreatment on plasma endotoxin level during I/R injury

In the second series of experiments, plasma endotoxin level was evaluated after intestinal I/R injury. There was a statistically significant difference in plasma endotoxin level between the control (group Ⅰ), and intestinal I/R injury (group Ⅲ) groups (0.76 EU/mL *vs* 1.64 EU/mL, $P < 0.001$). Enteral Gln pretreatment lowered the plasma endotoxin level (group Ⅳ), which was increased due to intestinal I/R injury (group Ⅲ) (1.54 EU/mL *vs* 1.64 EU/mL, $P = 0.48$). However, this decrease failed to reach statistical significance. There was no significant difference in plasma endotoxin levels between the control (group Ⅰ), and Gln (group Ⅱ) groups (0.76 EU/mL *vs* 0.86 EU/mL, $P = 0.59$) (Figure 2).

Effect of Gln pretreatment on intestinal histopathological changes during I/R injury

Intestinal histopathological changes were also evaluated in the second series of experiments. There was significant intestinal injury due to intestinal I/R when the control group was compared to the I/R group (0 *vs* 3, $P < 0.001$). Gln pretreatment did not significantly decrease intestinal injury compared to the I/R group (3 *vs* 2, $P = 0.389$). There was no significant difference in histopathological changes between the control and Gln groups (Figure 3).

DISCUSSION

Ischemia and subsequent reperfusion is one of the major causes of cell injury. The mechanisms of I/R injury are complex, and are likely to differ with respect to the duration of ischemia, the specific tissue involved, and the species studied[3,28]. The small intestine may experience ischemia and reperfusion during septic shock[29], hemorrhagic shock[30] or cardio-vascular surgery[31,32]. These clinical situations can result in some serious postoperative complications such as delay in anastomotic healing[33]. Intestinal mucosa is known to be sensitive to I/R injury. Its basal high rate of oxygen use renders the intestine relatively incapable of increasing oxygen transport in cases of hypoxic stress, and thus is

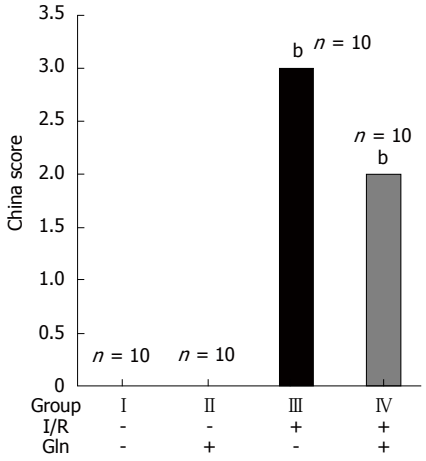


Figure 3 The effect of enteral Gln pretreatment (1 g/kg per day for four days) on histopathological changes during I/R injury in rats. Intestinal I/R was induced by 60 min of occlusion, followed by 60 min of reperfusion. Histopathological changes were scored by the "Chiu" intestinal ischemia scoring system. Data are expressed as medians. $^{b}P < 0.001$ *vs* groups Ⅰ and Ⅱ.

more susceptible to ischemic injury. Intestinal ischemia predisposes the gut to subsequent necrosis. Restoration of blood flow and reintroduction of oxygen after deprivation accelerates tissue injury[34]. Reperfusion of ischemic intestine may lead to more severe functional and morphological changes than the injury produced by ischemia itself[3]. Oxygen free radicals are thought to be generated during the reperfusion phase, with the addition of oxygen to previously ischemic tissues causing the formation of reactive oxygen species[35].

Intestinal I/R disrupts the functional intestinal mucosal barrier, which results in an increase in mucosal permeability to ^{51}Cr-EDTA[5]. The results of the present study also demonstrated a significant increase in intestinal permeability to ^{51}Cr-EDTA, due to intestinal I/R injury (Figure 1). An increase in mucosal permeability also promotes bacterial translocation[36]. Bacteria colonizing the gastrointestinal tract can translocate from the intestinal lumen to the bloodstream, and this may cause both systemic infection and/or infections in distant organs, such as mesenteric lymph nodes, spleen and liver[9]. Intestinal I/R injury does not only result in bacterial translocation, but also promotes an increase in plasma endotoxin level[6,37]. Our study also demonstrated a significant increase in plasma endotoxin level parallel to disruption of intestinal tissue in the intestinal I/R group when compared to the control group. Bacterial translocation and increased levels of endotoxin in the circulation may initiate a systemic inflammatory response, and the secretion and activation of inflammatory mediators, including cytokines[38] and metabolites of arachidonic acid[39]. An increase in intestinal permeability, and the deleterious effect of endotoxin in non-steroidal anti-inflammatory drug (NSAID)-induced ulcers has also been observed in experimental NSAID-induced enteropathy[40]. Gut decontamination decreases plasma endotoxin levels, and attenuates the systemic injury in intestinal I/R in rats[11]. A beneficial effect of antibiotics was also observed in NSAID-induced enteropathy[41,42].

The results of our study demonstrated that glutamine pretreatment prevented the increase in intestinal permeability during intestinal I/R, which has been reported previously[6,43]. However, glutamine pretreatment itself did not change the intestinal permeability significantly when compared to that in the control group. It has previously been shown that administration of glutamine-enriched nutritional support protects the bowel from injury due to abdominal irradiation[44], chemotherapy[45] and sepsis[46].

Glutamine pretreatment also caused a decrease in plasma endotoxin level, which did not reach statistical significance in our study. Contrary to our result, Wu *et al* have reported that an increase in plasma endotoxin level due to intestinal I/R can be reduced by Gln-supplemented total parenteral nutrition[6]. However, our study differed from that of Wu *et al* regarding the dose, route and timing of Gln administration. Furthermore, Gln was delivered in a total parenteral nutrition solution containing various amino acids and lipids in the study of Wu *et al*. Entrance of ^{51}Cr-EDTA and endotoxin to the systemic circulation from the intestinal lumen may be driven by different mechanisms, which may also explain differential effects of Gln on the above-mentioned parameters. The possibility that colonic epithelial cells contain specific transport systems for endotoxin has been reported[47]. Intestinal I/R disrupted intestinal tissue significantly. Gln pretreatment did not prevent histopathological disruption. It has also been observed that there is no correlation between histopathological alterations and intestinal permeability during recovery after hemorrhagic shock which is accepted as a model of intestinal I/R[30].

In conclusion, although Gln pretreatment reversed increased intestinal permeability, it did not prevent an increase in plasma endotoxin levels or histopathological alterations in intestinal I/R. Further studies are necessary to clarify the effects of different doses and administration periods of Gln on plasma endotoxin levels and histopathological changes in intestinal I/R.

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COMMENTS

Background

Impairment of microcirculation during I/R in the gastrointestinal tract may diminish intestinal mucosal integrity and cause an increase in intestinal permeability. Increased plasma endotoxin levels after I/R are a major threat to many surgical patients. Different Gln treatments are known to have protective effects on mucosal integrity in intestinal I/R.

Research frontiers

Injury to the intestinal barrier results in an increase in permeability to intraluminal substances. Correlation between morphological alterations and the degree of increased intestinal permeability is uncertain. Recent studies have demonstrated that morphological alterations after intestinal mucosal injury cannot reflect the function of the intestinal barrier.

Innovations and breakthroughs

Recent studies suggest that gastrointestinal epithelial cells contain specific transport systems for lipopolysaccharides.

Applications

Intestinal permeability can be measured by many different *in vitro* and *in vivo* methods. Endotoxin passing through the intestinal mucosa into the circulation indicates loss of the intestinal barrier function. Measurement of plasma endotoxin level ensures the assessment of real pathogenic factors that arise in the systemic circulation as a result of intestinal mucosal injury.

Terminology

Bacterial translocation: indigenous bacteria that colonize the gastrointestinal tract can cross the epithelial mucosa to infect distant organs. Under normal conditions, the epithelial lining prevents the escape of these bacteria from the gut lumen. Intestinal permeability: relates to the properties and function of the epithelial barrier that enables unmediated passage of substances through the intestinal mucosa. The use of intestinal permeability tests for screening of intestinal disease and assessment of treatment efficacy, to understand the normal intestinal physiology and pathogenesis of disease, is described and reviewed. There is now a need for research into the basic mechanisms of regulatory control of the intestinal barrier function.

Peer review

Peer reviewers considered this to be a very interesting paper with a great deal of potential clinical benefit. These basic results may be of value in clinical applications in organ or cellular transplantion.

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RAPID COMMUNICATION

Single-center study comparing computed tomography colonography with conventional colonoscopy

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Abstract

AIM: To compare the results from computed tomography (CT) colonography with conventional colonoscopy in symptomatic patients referred for colonoscopy.

METHODS: The study included 227 adult outpatients, mean age 60 years, with appropriate indications for colonoscopy. CT colonography and colonoscopy were performed on the same day in a metropolitan teaching hospital. Colonoscopists were initially blinded to the results of CT colonography but there was segmental unblinding during the procedure. The primary outcome measures were the sensitivity and specificity of CT colonography for the identification of polyps seen at colonoscopy (i.e. analysis by polyp). Secondary outcome measures included an analysis by patient, extracolonic findings at CT colonography, adverse events with both procedures and patient acceptance and preference.

RESULTS: Twenty-five patients (11%) were excluded from the analysis because of incomplete colonoscopy or poor bowel preparation that affected either CT colonography, colonoscopy or both procedures. Polyps and masses (usually cancers) were detected at colonoscopy and CT colonography in 35% and 42% of patients, respectively. Of nine patients with a final diagnosis of cancer, eight (89%) were identified by CT colonography as masses (5) or polyps (3). For polyps analyzed according to polyp, the overall sensitivity of CT colonography was 50% (95% CI, 39%-61%) but this increased to 71% (95% CI, 52%-85%) for polyps $\geqslant$ 6 mm in size. Similarly, specificity for all polyps was 48% (95% CI, 39%-58%) increasing to 67% (95% CI, 56%-76%) for polyps $\geqslant$ 6 mm. Adverse events were uncommon but included one colonic perforation at colonoscopy. Patient acceptance was high for both procedures but preference favoured CT colonography.

CONCLUSION: Although CT colonography was more sensitive in this study than in some previous studies, the procedure is not yet sensitive enough for widespread application in symptomatic patients.

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Key words: Colorectal polyps; Colorectal cancer; Computed tomography colonography; Colonoscopy

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INTRODUCTION

CT colonography is a newer radiological procedure that might be suitable for screening for colorectal lesions in symptomatic and asymptomatic individuals[1-6]. An obvious consideration is the sensitivity, specificity and accuracy of the investigation in different patient populations. A second consideration is the frequency of colorectal abnormalities on CT colonography that will require subsequent colonoscopy. Ideally, this percentage should be relatively low. Yet a third consideration is the cost of the procedure in money and time and the frequency of minor and more serious complications.

In previous studies that have compared CT colonography

with colonoscopy, the sensitivity, specificity and accuracy of CT colonography has shown substantial variation. In one large screening study in asymptomatic adults, CT colonography had a similar sensitivity to colonoscopy for the detection of polyps greater than 6 mm in diameter[7]. In contrast, studies in symptomatic patients have shown a sensitivity for the detection of cancers of approximately 75% and lower sensitivities for the detection of polyps[8,9]. This variation may reflect differences in bowel preparation, colonic distension, CT scanners, collimation, software, scan evaluation and use of fecal tagging[10-13]. In this study, the comparison of CT colonography with colonoscopy included the routine use of hyoscine butylbromide (Buscopan) during CT colonography, unblinding of the results of CT colonography during colonoscopy and repeat colonoscopy for discrepant findings.

MATERIALS AND METHODS

The study included 227 patients with appropriate indications for colonoscopy. Symptoms included rectal bleeding in 77 patients, abdominal pain in 56 and a change in bowel habit in 41. In addition, some patients had a family history of colorectal cancer (51), previous colonic polyps (43) and a recent positive fecal occult blood test (30). Exclusion criteria included inflammatory bowel disease and major coexisting medical disorders. Patients had a mean age of 60 years with an age range of 25 to 85 years. There were similar numbers of men (51%) and women (49%). All patients gave informed written consent and the study was approved by the local institutional Ethics Committee in 2003.

CT colonography and colonoscopy were performed on the same day. The type of bowel preparation was determined by the colonoscopist but most patients had Picolax, Colonlytely or both preparations. CT colonography was performed with a Toshiba multislice helical CT scanner with 2 mm collimation that was reconstructed into intervals of 1.0-1.5 mm. Colonic distension was achieved by insufflation of carbon dioxide and the use of intravenous Buscopan (20 mg). Patients were scanned in both supine and prone positions during a single breath hold (average scanning time, 25 s). With this technique, the radiation dose is lower than that of a barium enema X-ray. Images obtained with a Compaq® PC using ColonScreen (Voxcar Ltd., Edinburgh) were read in 2-dimensional format with use of a targeted 3-dimensional format when necessary. The CT images were reported by one of three radiologists within 60 min of completion of the scan. All had received previous training in CT colonography and had been reporting CT colonography on a routine basis prior to the study. The adequacy of the bowel preparation was recorded and the presence and size of polyps and masses were reported for eight segments (caecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon and rectum).

Colonoscopy was performed by a consultant gastroenterologist or colorectal surgeon. Patients were sedated with intravenous fentanyl and midazolam, sometimes supplemented with propofol. The colonoscope was passed to the caecum and then slowly withdrawn to the splenic flexure. At that point, CT colonography findings in the right colon were made available to the colonoscopist and colonoscopy was repeated if there were discrepant results. This also applied for CT colonography findings in the left colon after withdrawal of the colonoscope to the rectum. Masses and polyps detected at colonoscopy were reported in eight segments as above.

The quality of the bowel preparation was recorded on a scale of 1-5 using the system of Yee *et al*[14]. Patient discomfort with both CT colonography and colonoscopy were assessed by a questionnaire soon after CT colonography and during recovery after colonoscopy. Patients also completed a similar questionnaire 1 wk after the procedures that included a question on preference for CT colonography or colonoscopy. All adverse events were recorded.

Statistical analysis

Comparisons of CT colonography with colonoscopy were analyzed according to polyp and according to patient using Stata Version 9. In the more important analysis according to polyp, confidence intervals were produced using logistic regression and reflect an allowance for clustering of polyps within patients. Polyps were judged to be identical if they were located in the same or adjacent segments and if they were of similar size (± 50%). In the analysis according to patient, exact confidence intervals were calculated by the Stata contributed program "diagt". Patients with at least one polyp identified by colonoscopy were a true positive if they had at least one polyp at CT colonography. It was not necessary for the lesions to be assessed as identical.

RESULTS

CT colonography was compared with colonoscopy in 202 of 227 patients. Twenty-five patients (11%) were excluded from the analysis because of incomplete colonoscopy or poor bowel preparation that largely affected CT colonography. Polyps and masses were detected at CT colonography and colonoscopy in 42% and 35% of patients, respectively.

Detection of cancer

Of the 9 patients with a final diagnosis of cancer, 8 were diagnosed at colonoscopy and confirmed by biopsy while 1 patient had cancer within a polyp that was diagnosed histologically. Cancers were located in the sigmoid colon (3), rectum (3), ascending colon (2) and splenic flexure (1). All of these lesions were seen at CT colonography but only 5 were considered as probable cancers. The remaining lesions were interpreted as polyps (3) and fecal material (1). Three additional patients were diagnosed with probable cancer at CT colonography but only 1 had a corresponding polyp at colonoscopy. Polyps and cancers detected at both CT colonography and colonoscopy are shown in Figure 1.

Detection of polyps

At colonoscopy, 163 polyps were detected in 69 patients. The most common sites were the sigmoid colon (30%), rectum (27%), transverse colon (22%) and ascending colon (12%). Histologically, the majority of polyps were

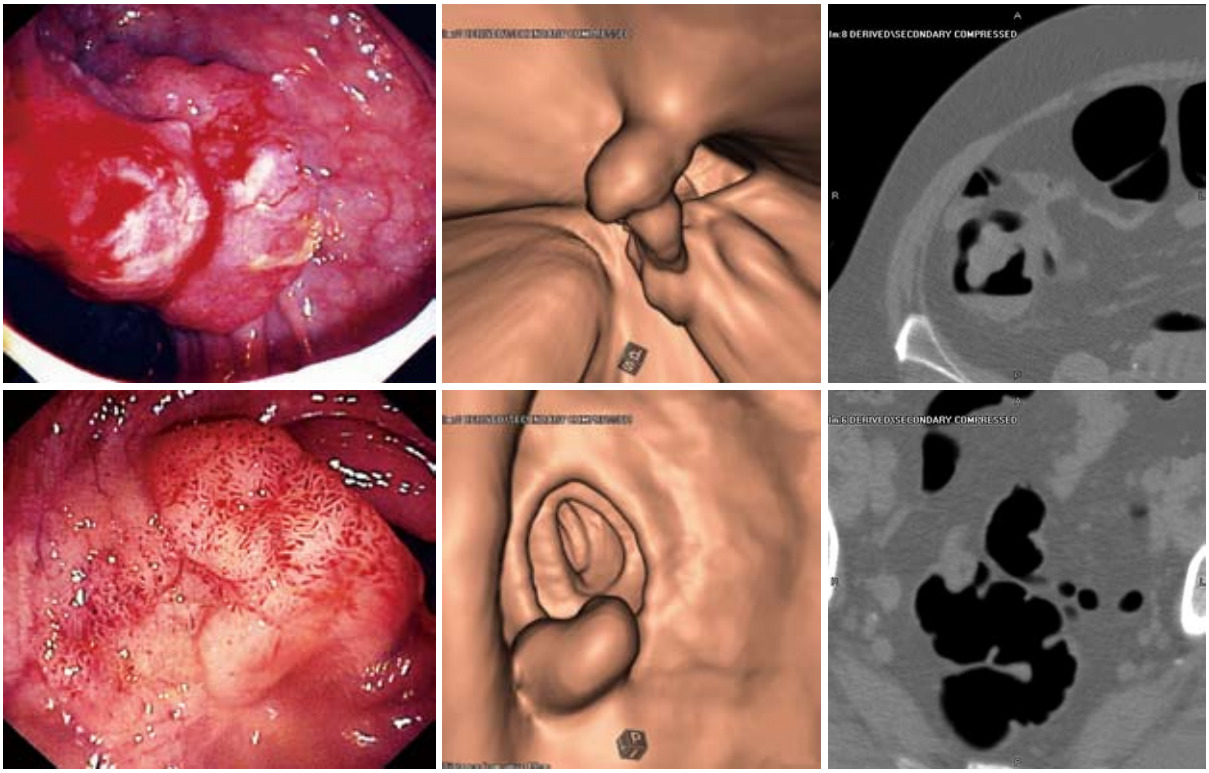


Figure 1 The figure shows the endoscopic appearance and images at CT colonography (3-D and 2-D) for 2 colonic lesions. The upper panel shows a cancer in the ascending colon while the lower panel shows a large tubulovillous adenoma at the rectosigmoid junction.

adenomatous (67%) while the remainder were hyperplastic, serrated or unspecified. In the analysis according to polyp, the sensitivity, specificity and accuracy of CT colonography for the detection of polyps of various sizes is shown in Table 1. The sensitivity for all polyps was 50% but this increased to 71% for polyps $\geqslant$ 6 mm in size. In the analysis according to patient, CT colonography had an overall sensitivity, specificity and accuracy of 62% (95% CI, 50%-74%), 76% (95% CI, 67%-83%), and 71% (95% CI, 64%-77%), respectively. For polyps $\geqslant$ 6 mm analyzed by patient, sensitivity, specificity and accuracy were 78% (95% CI, 58%-91%), 82% (95% CI, 76%-88%) and 82% (95% CI, 76%-87%). In relation to the sensitivity of colonoscopy, one polyp was detected by CT colonography that was missed at the initial colonoscopy but detected by repeat colonoscopy.

Extracolonic findings

Data on extracolonic findings were available in 225 of 227 patients (99%). One patient had a renal mass and was subsequently diagnosed with renal cell cancer. Other findings of potential clinical significance included renal stones (7%), gallbladder stones (6%), adrenal masses (4%), ovarian cysts (3%), non-cystic liver lesions (2%), small aortic aneurysms (2%), lung nodules (1%) and calcific chronic pancreatitis (1%).

Bowel preparation at CT colonography

Only 33% of patients had a clean bowel (grade 1) while 62% had "pools of liquid" (grade 2) and 5% had "solid and liquid" or "collections of solid feces" (grades 3 and 4). Patients categorized as "impossible" (grade 5) were excluded from the study.

Adverse events

These were reported by 4 patients after CT colonography and included nausea (2), dizziness (1) and significant abdominal pain (1). Adverse events after colonoscopy included rectal bleeding (1) and moderate or severe abdominal pain (2). One of these patients was subsequently diagnosed with colonic perforation and treated by resection of the sigmoid colon. There were no postoperative complications. Other recorded complications during colonoscopy included hypoxia (1), hypertension (1) and bradycardia (1).

Patient acceptance and preference

Selected results from a questionnaire administered 1 wk after the procedures are included in this report. The questionnaire was completed by 195 of 202 patients (96%). The experience of CT colonography and colonoscopy was "better than expected" or "as expected" in 84% and 94% of patients, respectively. Overall, 87% of patients were "very satisfied" or "satisfied" with CT colonography while 90% were "very satisfied" or "satisfied" with colonoscopy. Furthermore, 89% of patients indicated that they "definitely would" or were "most likely" to have repeat CT colonography compared to 96% for colonoscopy. These differences were not statistically significant. However, when asked to choose between CT colonography and colonoscopy for a repeat procedure, 61% chose CT colonography and 39% chose colonoscopy ($P = 0.005$, Chi-square test).

DISCUSSION

CT colonography, also called virtual colonoscopy, is an

Table 1 Sensitivity, specificity and accuracy of CT colonography when analyzed by polyp (%)

Polyp size	*n*	Sensitivity	Specificity	Accuracy
< 6 mm	125	42 (30-55)	63 (52-73)	54 (46-62)
6-10 mm	27	78 (52-92)	75 (65-83)	75 (66-83)
> 10 mm	11	62 (39-81)	86 (77-92)	82 (73-89)
All polyps	163	50 (39-61)	48 (39-58)	49 (42-56)
⩾ 6 mm	38	71 (52-85)	67 (56-76)	68 (59-76)

Values in brackets are 95% confidence intervals that reflect an allowance for clustering of polyps within patients.

evolving technology that may have a role in screening for colorectal polyps and cancer. However, using colonoscopy as the gold standard, concordance between the two investigations has varied widely in different studies. In relation to the patient population, better results have been achieved in screening studies in asymptomatic individuals than in patients with symptoms. This may reflect the younger age and better health of asymptomatic individuals who are more able or more motivated to comply with bowel preparation procedures. In any event, the quality of the bowel preparation is more important for CT colonography than for colonoscopy. In the present study, the adequacy of the bowel preparation was recorded by the reporting radiologist. Only 33% of patients had a "clean bowel" while the remainder had either "pools of liquid" (62%) or "solid and liquid" (5%). Whether bowel images can be improved by modified preparation protocols[15] or by "electronic cleansing"[16] remains unclear. Intravenous Buscopan is not routinely used during CT colonography but does enhance colonic distension[17].

Other factors that may contribute to variable results from CT colonography include the type and settings of CT scanners, the use of fecal tagging, the mode of imaging and the experience of the reporting radiologist. In one meta-analysis[10], better results were achieved with scanners with multiple detectors, thinner collimation and the standard use of three-dimensional ("fly-through") technology. Fecal tagging using oral contrast did not appear to be a relevant factor and has the potential to impair the quality of colonoscopy. When images are reported by different radiologists, variation has ranged from "minimal"[7] to "substantial"[18], apparently independent of previous experience with the technique. There are also potential biases in comparative studies of this type with over-reporting in the investigation under study, specifically CT colonography.

The experience of both CT colonography and colonoscopy was widely accepted by patients. Although responses to specific questions about each technique showed a trend in favour of colonoscopy, more patients chose CT colonography when given a choice between the two procedures. These responses can be influenced by the structure of the questions but might also reflect real concerns about sedation, potential complications and the degree of "invasiveness". In this study, complications were uncommon but one patient had a colonic perforation that required surgery. This complication has an overall frequency of approximately 1 in 1000 colonoscopies[19,20] but can also occur after CT colonography, perhaps with a frequency of 1 in 1700 procedures[21].

In this study, CT colonography was more sensitive than in previous studies in symptomatic patients[8,9]. For example, CT colonography identified cancers as either masses or polyps in 8 of 9 patients (89%) in this study and in 6 of 8 (75%)[8] and 7 of 9 (78%)[9] in previous studies. For polyps analyzed by polyp, the overall sensitivity for detection of polyps was 50% in this study increasing to 71% for polyps 6 mm in size or greater. In previous studies, sensitivities for polyp detection (⩾ 6 mm) were reported as 32%[8] and 47%[9]. The possibility that inaccurate localisation of polyps at colonoscopy contributed to low sensitivity rates seemed unlikely as sensitivities for polyps ⩾ 6 mm analyzed by patient were only marginally higher (78%) than those analyzed by polyp (71%).

Criteria for the introduction of new technologies into clinical practice are difficult to establish. For example, issues such as sensitivity and specificity need to be considered in relation to direct and indirect costs, complications and the proportion of patients who will be referred for further investigation. In the present study, CT colonography identified polyps or masses in 42% of patients. This is not a cost-effective option if all patients with abnormalities are referred for colonoscopy[22]. However, CT colonography becomes increasingly cost-effective if colonoscopy is restricted to lesions that are ⩾ 6 mm or ⩾ 10 mm in size. Such recommendations, if introduced, would invite a new series of questions related to the frequency of repeat scans and the mutagenic effects of accumulating doses of radiation[23-25]. Although small polyps have a low frequency of features of advanced neoplasia, there is only limited data on the natural history of small polyps and the identification of those polyps that will eventually evolve into cancer[7,26].

Although CT colonography is not yet ready for widespread clinical application, it is likely that results will improve with better bowel preparation, technical developments and increasing familiarity with the technique. Results from this study highlight the importance of bowel preparation although, in the future, it may be possible to reliably differentiate fecal material from polyps using fecal tagging[10], "electronic cleansing" of colonic fluid[16] or contrast-enhanced studies[27]. Other helpful developments may also include new fecal subtraction algorithms[28], workstation modifications to facilitate polyp detection[29], improved software and new training programs for radiologists[30].

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COMMENTS

Background

It is not yet clear whether computed tomography (CT) colonography will be widely adopted for the detection of colonic polyps and cancer. Current issues include

the sensitivity and specificity of the investigation, direct and indirect costs and the longer-term effects of radiation.

Research frontiers

This study indicates that residual liquid and solid material after bowel preparation limits the accuracy of the procedure. Whether this can be overcome by modified laxative preparations, fecal tagging or "electronic" bowel cleansing remains unclear.

Innovations and breakthroughs

In this study in symptomatic patients, CT colonography was more sensitive for the detection of polyps than in some previous studies. This study included the routine use of intravenous Buscopan but the study was not designed to determine whether this modification improved either sensitivity or specificity.

Applications

There is widespread interest in screening for colon cancer in symptomatic and asymptomatic individuals. Colonography, either using CT or magnetic resonance imaging, may play a central role if there are further improvements in sensitivity and specificity.

Peer review

The paper by Roberts-Thomson IC *et al* is interesting. The selection of patients, sample size and the overall design of the study are fair, the results adequate to provide clinical evidence and to support valid conclusions.

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RAPID COMMUNICATION

Impact of alanyl-glutamine dipeptide on severe acute pancreatitis in early stage

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Abstract

AIM: To evaluate the therapeutic effect of alanyl-glutamine dipeptide (AGD) in the treatment of severe acute pancreatitis (SAP) in early and advanced stage.

METHODS: Eighty patients with SAP were randomized and received 100 mL/d of 20% AGD intravenously for 10 d starting either on the day of (early treatment group) or 5 d after (late treatment group) admission. Groups had similar demographics, underlying diseases, Ranson score, Acute Physiology and Chronic Health Evaluation Ⅱ (APACHE Ⅱ) score, and Balthazar's computed tomography (CT) score at the beginning of the study and underwent similar other medical and nutritional management.

RESULTS: The duration of acute respiratory distress syndrome (2.7 ± 3.3 d *vs* 12.7 ± 21.0 d, $P < 0.01$), renal failure (1.3 ± 0.5 d *vs* 5.3 ± 7.3 d, $P < 0.01$), acute hepatitis (3.2 ± 2.3 d *vs* 7.0 ± 7.1 d, $P < 0.01$), shock (1.7 ± 0.4 d *vs* 4.8 ± 3.1 d, $P < 0.05$), encephalopathy (2.3 ± 1.9 d *vs* 9.5 ± 11.0 d, $P < 0.01$) and enteroparalysis (2.2 ± 1.4 d *vs* 3.5 ± 2.2 d, $P < 0.01$) and hospital stay (28.8 ± 9.4 d *vs* 45.2 ± 27.1 d, $P < 0.01$) were shorter in the early treatment group than in the late treatment group. The 15-d APACHE Ⅱ score was lower in the early treatment group than in the late treatment group (5.0 ± 2.4 *vs* 8.6 ± 3.6, $P < 0.01$). The infection rate (7.9% *vs* 26.3%, $P < 0.05$), operation rate (13.2% *vs* 34.2%, $P < 0.05$) and mortality (5.3% *vs* 21.1%, $P < 0.05$) in the early treatment group were lower than in the late treatment group.

CONCLUSION: Early treatment with AGD achieved a better clinical outcome in SAP patients.

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Key words: Severe acute pancreatitis; Alanyl-glutamine dipeptide; Clinical study

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INTRODUCTION

Acute pancreatitis (AP) contributes to thousands of annual hospital admissions, of which severe acute pancreatitis (SAP) accounts for 10%-20%[1-3]. Despite considerable improvement in the treatment, the mortality of SAP still ranges between 10%-15%[2-5].

The course of SAP tends to be prolonged and the patients usually are hypermetabolic and high protein catabolic due to systemic inflammatory response syndrome (SIRS) induced by acute local inflammatory process and subsequent vital-organ dysfunction[6]. Thus, if nutritive support is not appropriately administrated to match rapidly increased demand in the treatment of SAP, the patients consequently come down with metabolic disorder and nutrition deficiency, which is considered to increase mortality due to impaired immune function, increased risk of infections and intractable vital-organ failure.

In recent years, research showed a conditional deficiency of glutamine would be an independent predictive factor for a poor outcome and its correction improved survival by restoring cellular protective mechanisms, improving immune function and resistance of the gut barrier to hypoperfusion, metabolic stress and subsequent bacterial translocation, and decreasing the risk of infection in critical illness[7-10]. Since free glutamine is instable in solution, intravenous administration is limited[7]. Alanyl-glutamine

dipeptide (AGD), however, can be taken *via* vein and hydrolyzed into alanine and glutamine in circulation as a substitute[7]. Presently AGD supplement in parenteral nutrition is a worth-trying approach and an evidence-based recommendation in the management of SAP[11], but there has been no study describing an optimal protocol of AGD administration. Our study aims to evaluate the favorable effects of early supplement with AGD in the treatment of SAP.

MATERIALS AND METHODS

Patient selection

In this study, the diagnostic criteria[12] formulated for SAP at the Bangkok World Congress of Gastroenterology 2002 in Thailand was employed. All of the patients, who had been diagnosed with SAP and admitted to our hospital within 72 h after onset of symptoms, were included. The patients who had histories of trauma, operation or prior treatment with AGD were excluded, and the patients who died within 5 d after admission were also rejected.

Methods

In this study, 80 identified SAP patients who were admitted to West China Hospital of Sichuan University from May 2004 to March 2005 were randomized and treated with 100 mL/d of 20% AGD intravenous infusion for 10 d (SSPC No. SF1505) starting either on the day of (early treatment group) or 5 d after (late treatment group) admission.

Upon admission, all of the patients were treated with intensive care, oxygen inhalation, intermittent gastrointestinal decompression, and fluid infusion. Prophylactic antibiotics were used for 7-14 d. H2 receptor antagonist or proton pump inhibitor agent was given for 7 d; the acid-base balance and the electrolyte balance were maintained. When patients developed respiratory failure, the respirator was employed to assist respiration. When hypoalbuminaemia occurred, 20% human serum albumin 50 mL was used daily until the serum albumin was recovered to normal, and fat emulsion was also given for 14 d.

The following parameters were measured: 24-h APACHE Ⅱ score and initial Balthazar's CT score, 15-d APACHE Ⅱ score, incidence and duration of complications including acute respiratory distress syndrome (ARDS), renal failure, acute hepatitis, encephalopathy and enteroparalysis, infection rate, hospital stay, operation rate and mortality.

Hospital stay: Hospital stay was defined as the duration from hospital admission to discharge. The duration of hospital rehabilitation due to cholecystectomy was not taken into account in this study, although cholecystectomy was regarded as a promising treatment to prevent recurrent pancreatitis.

Operation rate: Surgical intervention was performed if infected pancreatic necrosis, or pancreatic abscess, or (per)pancreatic hemorrhage or (per)pancreatic pseudocyst was identified or if the patient did not respond to intensive care treatment.

Table 1 Baseline in the two groups

Baseline	Late treatment group (n = 38)	Early treatment group (n = 38)
Sex (Male/Female)	21/17	22/16
Age (mean ± SD, yr)	47.5 ± 12.6 (22-76)	46.9 ± 12.8 (27-74)
Etiology, n (%)		
Gallstones	20 (52.6)	16 (42.1)
Alcohol abuse	2 (5.3)	7 (18.7)
Hyperlipidemia	7 (18.4)	9 (23.7)
Idiopathic	9 (23.7)	6 (15.8)
48-h Ranson score (mean ± SD)	4.5 ± 1.7	4.8 ± 1.6
24-h APACHE Ⅱ score (mean ± SD)	10.8 ± 3.5	10.2 ± 3.1
CT score (mean ± SD)	5.8 ± 2.3	5.9 ± 2.4

APACHE Ⅱ: Acute Physiology and Chronic Heath Evaluation Ⅱ; CT: Computed Tomography.

Statistical analysis

Data were expressed as mean ± SD or percentage. Data in normal distribution was analyzed using t test; data in non-normal distribution was analyzed using Wilcoxon rank sum test. Categorical data was analyzed using Chi-square test. $P < 0.05$ was considered statistically significant.

The medical ethics committee of West China Hospital at Sichuan University approved this study. All patients gave their informed consent, and the study was conducted according to the recent principles of the Declaration of Helsinki (World Medical Association, 2000).

RESULTS

Four patients including 1 death within 24 h after admission and 1 death on the 5th day after admission in the early treatment group and 2 deaths within 24 h in the late treatment group withdrew from the study, which were not included in any of the analyses. Therefore, there were 38 patients in the early treatment group and 38 in the late treatment group.

Baseline

There were no statistical differences between the two groups in sex, age and etiology ($P > 0.05$, Table 1), and in the 48-h Ranson score, 24-h APACHE Ⅱ score and CT score in the initial stage of hospitalization ($P > 0.05$, Table 1).

Complications

There were no statistical differences between the two groups in the incidences of ARDS, renal failure, shock, acute hepatitis, encephalopathy and enteroparalysis ($P > 0.05$), but the duration of ARDS, renal failure, acute hepatitis, encephalopathy and enteroparalysis was shorter in the early treatment group than in the late treatment group ($P < 0.01$), and the duration of shock was also shorter in the early treatment group ($P < 0.05$) (Table 2).

Prognosis

The 15-d APACHE Ⅱ score was lower in the early treatment group than in the late treatment group ($P < 0.01$).

Table 2 Incidence and duration of complications in the two groups (*n*, %) (mean ± SD)

Complications	Late treatment group (*n* = 38)		Early treatment group (*n* = 38)	
	Cases (%)	Duration (d)	Cases (%)	Duration (d)
ARDS	18 (47.4)	12.7 ± 21.0	21 (55.3)	2.7 ± 3.3[b]
Renal failure	9 (23.7)	5.3 ± 7.3	7 (18.4)	1.3 ± 0.5[b]
Acute hepatitis	19 (50.0)	7.0 ± 7.1	17 (44.7)	3.2 ± 2.3[b]
Shock	6 (15.8)	4.8 ± 3.1	9 (23.7)	1.7 ± 0.4[a]
Encephalopathy	4 (10.5)	9.5 ± 11.0	4 (10.5)	2.3 ± 1.9[b]
Enteroparalysis	27 (71.1)	3.5 ± 2.2	29 (76.3)	2.2 ± 1.4[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* late treatment group; ARDS: acute respiratory distress syndrome.

Table 3 Fifteen-day APACHE Ⅱ score, hospital stay (d), infection, operation and mortality in the two groups

Prognosis	Late treatment group (*n* = 38)	Early treatment group (*n* = 38)
15-d APACHE Ⅱ score (mean ± SD)	8.6 ± 3.6	5.0 ± 2.4[b]
Hospital stay (d, mean ± SD)	45.2 ± 27.1	28.8 ± 9.4[b]
Infection, *n* (%)	10 (26.3)	3 (7.9)[a]
Operation, *n* (%)	13 (34.2)	5 (13.2)[a]
Deaths, *n* (%)	8 (21.1)	2 (5.3)[a]

[a]$P < 0.05$, [b]$P < 0.01$.

The infection rate, operation rate and mortality were also lower in the early treatment group ($P < 0.05$). The hospital stay was shorter in the former group than in the latter group ($P < 0.01$) (Table 3).

DISCUSSION

In the early stage of SAP, the patients tend to be hypermetabolic due to occurrence of SIRS and subsequent multiple organ dysfunction syndromes (MODS), resulting in the greatly increased demand for nutrition[13-15]. In the late stage, the demand for nutrition increases continuously due to infection, resulting from intestinal bacterial translocation and immunosuppression. Thus, insufficient nutritive support inevitably leads to nutrition deficiency in SAP patients[16].

When a nutritional deficiency arises in critical illness including SAP, glutamine, which is very abundant and readily synthesized under most situations, tends to be a conditional depletion[17]. The low concentration of plasma glutamine was found to be an independent predictive factor for a poor outcome in critical illness[18].

AGD was shown to improve clinical outcome of SAP[11,16]. In this study, we treated SAP patients with 100 mL/d of 20% AGD infusions intravenously for 10 d to compare the effects of AGD between the two groups and study its optimal protocol. The baseline data showed no significant difference between the two groups ($P > 0.05$). APACHE Ⅱ score, the parameter for predicting and monitoring the development of local and systemic complications of SAP, was evaluated on the 15th day after admission, which was lower in the early treatment group than in the late treatment group ($P < 0.01$). The duration of ARDS, renal failure, acute hepatitis, shock, encephalopathy and enteroparalysis were also shorter in the early treatment group than in the late treatment group, as was lower mortality ($P < 0.05$). These might result from a possible consequence of early suppression of inflammatory response and restoring cellular protective mechanisms by early AGD supplementation, which is associated with mediating anti-inflammatory/immunologic factors[19] and antioxidant/inducible nitric oxide synthase (iNOS)[20], decreasing the level of TNF-alpha and interleukin-8 in mononuclear cell[21,22] and C-reactive protein in serum[23]. In this study, the hospital stay was also shorter in the early treatment group ($P < 0.05$), which might be correlated to the shorter duration of complications.

Intestinal tract, the greatest "storeroom" of microorganisms, also is one of the most possible injury organs besides pancreas itself[24]. Intestinal bacterial translocation resulting from the impaired mucosal barrier, microorganism barrier, chemical barrier and immune barrier is an early event[25] and the main cause of infection in SAP[26]. The infection rate of SAP was 30%-50%[27-30], leading to a mortality of 40%-80%[31,32]. Glutamine is used as a major fuel and nucleotide substrate for rapidly dividing cells such as intestinal mucosal cells and the gut-associated immunocytes[33-37]. Glutamine can prevent atrophy of the intestinal epithelial cells through HSP 70 generation[38] and improve the intestinal immune barrier[39-41]. A meta-analysis by Novak F *et al*[42] revealed that glutamine could reduce the infectious morbidity (RR 0.84, 95% CI: 0.68-1.03) and mortality (RR 0.76, 95% CI: 0.56-0.98) in critical illness. Our study showed that early treatment with AGD in SAP patients could shorten the duration of enteroparalysis ($P < 0.01$) and reduce the infection rate ($P < 0.05$). This result may indicate that early nutrition with AGD in SAP patients could timely protect and restore the function of intestinal tract, shorten the duration of enteroparalysis, lower the risk of bacterial translocation, thus reducing the infection rate.

In conclusion, early treatment with AGD yields a better clinical outcome in SAP patients by reducing the duration of vital-organ dysfunction, decreasing the risk of infection and operation, and lowering the mortality.

COMMENTS

Background

Severe acute pancreatitis (SAP) is a hypermetabolic disease. Appropriate nutrition support is essential to the management of SAP. In recent years, the supplement of glutamate, the most abundant free amino acid playing vital roles in the body, has been shown to improve survival rate. Presently, the supplement of alanyl-glutamine dipeptide, as a substitute of glutamate which is stable in circulation, is a promising and worth-trying approach.

Research frontiers

Besides nutritional management of SAP, restoring an optimized immune system plays a role in improving survival rate. Previous pilot studies of alanyl-glutamine dipeptide (AGD) supplementation in nutritive support have revealed good outcome by restoring cellular protective mechanisms, improving the immune function and lowering the infection rate. Although this treatment was recommended by evidence-based studies, this treatment principle has not yet been systematically applied and further study is still needed in this field.

Innovations and breakthroughs

The optimal protocol for the AGD treatment is not yet available in published

studies. Early AGD treatment in SAP is a breakthrough in this study, which reveals for the first time that early AGD treatment achieved a better clinical outcome in SAP patients.

Applications

As free glutamine is instable in solution, intravenous administration is limited. AGD, however, can be given *via* vein and hydrolyzed into alanine and glutamine in circulation as a substitute. This study showed that early treatment with AGD achieved a better clinical outcome in SAP patients. This treatment can be applied in the management of SAP patients.

Terminology

Severe acute pancreatitis is a common acute abdominal disorder, characterized by various degrees of necrosis of pancreatic parenchyma together with local and systemic complications, such as SIRS and multiple organ dysfunction syndromes. Alanyl-glutamine dipeptide is an important component of parenteral nutrition ingredients with its molecular formula ingredient as N (2)-L-alanyl-L-glutamine. SIRS is a clinical response to one of the nonspecific insults caused by ischemia, inflammation, trauma, infection, or a combination of several insults, which was defined by a journal in 1992 and described as occurrence of two or more clinical symptoms of fever or hypothermia, tachypnea, tachycardia, and leukocytosis.

Peer review

AGD supplementation has been shown to be effective by previous studies. In this pilot study, the authors focused on the clinical effects of early treatment with AGD comparing with late treatment, which shows early AGD treatment indicates a better clinical outcome. Further researches are needed to explore its mechanism.

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RAPID COMMUNICATION

Comparison of real-time polymerase chain reaction with the COBAS Amplicor test for quantitation of hepatitis B virus DNA in serum samples

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Abstract

AIM: To compare the clinical performance of a real-time PCR assay with the COBAS Amplicor Hepatitis B Virus (HBV) Monitor test for quantitation of HBV DNA in serum samples.

METHODS: The reference sera of the Chinese National Institute for the Control of Pharmaceutical and Biological Products and the National Center for Clinical Laboratories of China, and 158 clinical serum samples were used in this study. The linearity, accuracy, reproducibility, assay time, and costs of the real-time PCR were evaluated and compared with those of the Cobas Amplicor test.

RESULTS: The intra-assay and inter-assay variations of the real-time PCR ranged from 0.3% to 3.8% and 1.4% to 8.1%, respectively. The HBV DNA levels measured by the real-time PCR correlated very well with those obtained with the COBAS Amplicor test (r = 0.948). The real-time PCR HBV DNA kit was much cheaper and had a wider dynamic range.

CONCLUSION: The real-time PCR assay is an excellent tool for monitoring of HBV DNA levels in patients with chronic hepatitis B.

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Key words: COBAS Amplicor test; Hepatitis B virus; Viral DNA; Real-time PCR

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INTRODUCTION

It is estimated that 350 million individuals are chronically infected with hepatitis B virus (HBV) worldwide, and about one-third of these live in China[1]. HBV infection can cause chronic hepatitis, cirrhosis and hepatocellular carcinoma[2]. Among patients with active viral replication, cirrhosis will develop in 15%-20% within 5 year[3], and 70-90 percent of cases of hepatocellular carcinoma occur against a background of cirrhosis[4]. Quantitation of HBV DNA in serum or plasma has been an important tool in identifying individuals with active hepatitis B, to monitor the efficiency of antiviral treatment, and to predict whether the treatment will be successful[5-9].

Several commercial molecular assays have been developed for quantitation of HBV DNA in serum or plasma samples. One of these assays is the COBAS Amplicor HBV Monitor, which is based on the amplification of DNA targets by the use of HBV-specific primers. Other methods, such as the VERSANT HBV DNA 3.0 assay, which is based on branched-DNA signal amplification, and Hybrid Capture Ⅱ, which is based on hybridization of a chemiluminescent probe, are also routinely used in diagnostic laboratories[10,11]. However, the costs of these assays are too high to be used in developing countries such as China, and in these countries the HBV burden is much heavier than in developed countries.

Recently, quantitative real-time PCR has been used to detect and quantify HBV levels in serum or plasma samples. This assay is based on the relationship between the initial DNA target levels and the threshold cycle (Ct) of the amplification products. The Ct value is smaller when the initial DNA target level is higher. Several evaluation studies have shown that real-time PCR has a higher sensitivity, a broader dynamic range, and an accurate quantitation of HBV DNA compared to those of the existing commercial methods[12-15].

The Fosun real-time PCR HBV kit is a commercial

assay for quantitation of serum HBV DNA levels based on TaqMan PCR technology. It has been approved by the State Food and Drug Administration (SFDA) of China for *in vitro* diagnostic use. This assay is widely used in Chinese laboratories for quantitative detection of serum HBV levels in patients with HBV infection. However, the clinical performance of this real-time PCR assay has not been compared with that of commercial assays routinely used in the United States and Europe. The aim of this study was to evaluate the clinical performance of the Fosun real-time PCR HBV assay on the ABI 7500 sequence detector for the quantitative detection of HBV DNA in serum samples. The results were compared to those obtained with the same samples using the COBAS Amplicor HBV Monitor system.

MATERIALS AND METHODS

Patients and samples

Serum samples from 158 individuals, including 118 patients with chronic hepatitis B (46 genotype B, 65 genotype C, and 7 mixed genotype B and C), and 40 blood donors, were analyzed in parallel by the Fosun real-time PCR HBV kit (Fosun Diagnostics, Shanghai, China) and COBAS Amplicor HBV Monitor (Roche Diagnostics). All the patients were positive for hepatitis B surface antigen (HBsAg) for at least 6 mo, and were negative for antibodies to hepatitis A, C, D and E viruses. None of these patients had received any antiviral treatment at the time of serum collection.

An HBV reference serum from the National Center for Clinical Laboratories of China with a DNA level of 7.5×10^8 copies/mL was used to determine the linearity and detection limit of the real-time PCR. This reference serum was 10-fold serially diluted into a spectrum of reference samples with HBV DNA levels ranging from 7.5×10^1 to 7.5×10^8 copies/mL.

A set of HBV reference sera established by the Chinese National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), with HBV DNA concentrations ranging from < 1000 to 6.17×10^8 copies/mL of genotype C were used for standardization.

Real-time PCR assay

For Fosun real-time PCR, HBV DNA was extracted from 100 μL serum by using the extraction reagents included in the kit, in accordance with the manufacturer's instructions. The amplification was performed on an ABI 7500 sequence detector (Applied Biosystems, Foster City, CA, USA) by incubating the reaction mixture (50 μL) at 50℃ for 2 min, 94℃ for 5 min, followed by 40 cycles of PCR amplification at 93℃ for 30 s and 60℃ for 90 s. The Ct value was defined as the number of fractional cycles in which the fluorescence emitted by the reaction mixture exceeded a preset threshold, and marked the beginning of an exponential growth of the fluorescence signal. The results were analyzed with ABI Prism software (Applied Biosystems). A four-point external standard set was used to calculate the initial copy number of the samples. The dynamic range of Fosun real-time PCR HBV DNA kits, as stated by the manufacturer, is 4.2×10^2-7.5×10^8 copies/mL.

COBAS Amplicor assay

Amplicor tests were performed by using the COBAS Amplicor system and the AMPLICOR HBV Monitor assay according to manufacturer's instructions. The highly conserved HBV precore/core region and the internal standard DNA were amplified in the same well, but hybridized in separate wells. The amount of HBV DNA was calculated from the ratio of the HBV well to the internal standard well, and the copy number per milliliter was calculated from a standard curve. The dynamic range of the Amplicor test was 3×10^2-2×10^5 copies/mL.

Statistical analysis

Statistical analyses were performed using the Statistical Program for Social Sciences (SPSS 13.0 for Windows; SPSS, Chicago, IL, USA). Correlation between the quantitative results from the two assays was determined with scatter plots and Spearman's coefficient analysis, after logarithmic transformation of data with skewed distribution. The Bland-Altman method was used to assess the agreement between the values obtained with the two assays[16].

RESULTS

Linearity and detection limit

The real-time PCR was able to detect seven (87.5%) of the eight dilutions of the reference serum. The tested values and the normal values of the dilutions were well matched ($r = 0.999$, $P < 0.001$). The converting formula was:

Log (HBV DNA level by real-time PCR) = 1.098 [Log (Reference HBV DNA level)] - 0.735

The real-time PCR was able to detect HBV DNA levels as low as 750 copies/mL, which is similar to the detection limit claimed by the manufacturer.

Inter-assay variation and accuracy of real-time PCR

To determine the dynamics and accuracy of the Fosun real-time PCR kit, the reference serum of the Chinese NICPBP was tested three times with kits with different lot numbers. Fosun real-time PCR kits detected all seven dilutions of the reference serum, and all the quantitative results were within the reference ranges (Table 1). The inter-assay variation ranged from 0.3% to 3.8%. The detection limit of the real-time PCR was 442 copies/mL.

Intra-assay variation and reproducibility of real-time PCR

For evaluation of the intra-assay variation of the Fosun real-time PCR kits, three samples with high, medium and low HBV titers were tested. Each sample was tested in 12 replicates. Table 2 shows the mean $\log_{10}$ (copies/mL) viral loads, SD and coefficient of variation (CV) of the samples. The viral load values from the Fosun real-time PCR assay were highly reproducible over the various HBV titers (CV, 1.4%-8.1%), especially for the samples with high and medium HBV titers, and the intra-assay CV was 1.4% and 2%, respectively.

Comparison of real-time PCR with COBAS Amplicor HBV Monitor

A total of 158 clinical serum samples were tested in

Table 1 Sensitivity of the Fosun real-time PCR HBV DNA assay and results of interassay testing

NICPBP references	Reference range (copies/mL)	Results (copies/mL)			Mean ($\log_{10}$ copies/mL)	SD ($\log_{10}$ copies/mL)	CV (%)
		Run 1	Run 2	Run 3			
L0	7.76×10^7 - 6.17×10^8	1.65×10^8	1.84×10^8	1.52×10^8	8.22	0.04	0.5
L1	1.48×10^7 - 1.18×10^8	9.21×10^7	1.01×10^8	9.23×10^7	7.98	0.02	0.3
L2	1.59×10^6 - 1.26×10^7	8.54×10^6	9.00×10^6	9.20×10^6	6.95	0.02	0.3
L3	1.66×10^5 - 1.32×10^6	7.06×10^5	6.13×10^5	9.55×10^5	5.87	0.10	1.7
L4	1.82×10^4 - 1.48×10^5	8.60×10^4	3.84×10^4	6.30×10^4	4.77	0.18	3.8
L5	1.51×10^3 - 1.23×10^4	1.07×10^4	6.20×10^3	8.82×10^3	3.92	0.12	3.1
L6	< 1000	524	442	581	2.71	0.06	2.2

Table 2 Intra-assay variation of the Fosun real-time PCR HBV DNA assay

No. of samples	Replicates	Mean of HBV DNA level ($\log_{10}$ copies/mL)	SD	CV (%)
3768	12	3.97	0.32	8.1
3785	12	6.00	0.12	2.0
3770	12	7.84	0.11	1.4

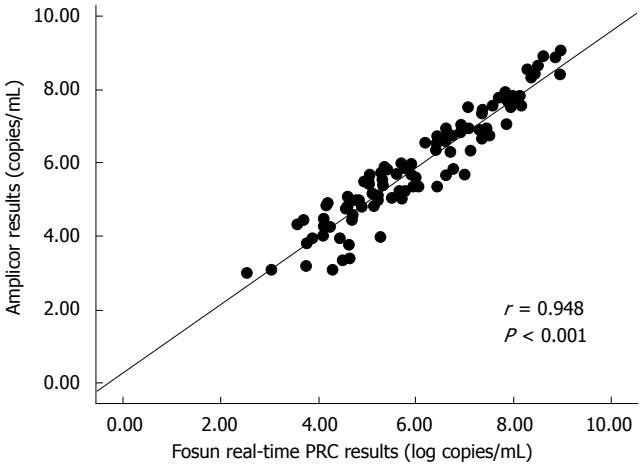


Figure 1 Correlation of HBV DNA levels measured by the Fosun real-time PCR and Amplicor tests.

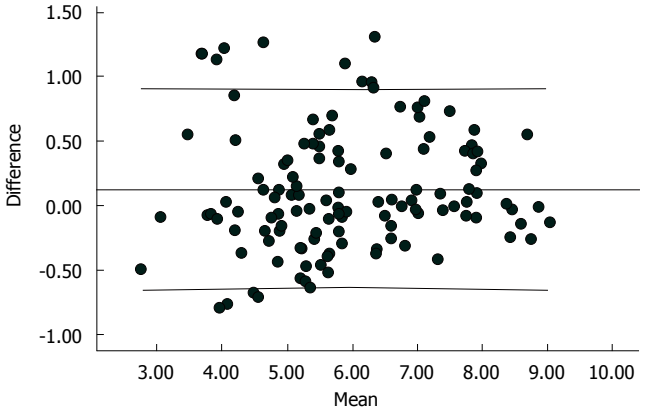


Figure 2 Differences between viral loads obtained by the two assays versus the average for each specimen.

parallel with the Fosun real-time PCR kits and the COBAS Amplicor HBV Monitor system. Samples were divided into three groups: group A, samples from healthy blood donors (n = 40); group B, samples from HBV infected patients with viral loads within the range of both tests (n = 43); group C, samples from HBV infected patients with viral loads above the upper limit of the Amplicor test (n = 75). These samples were diluted and re-tested with the Amplicor test. Viral loads were converted into logarithms. In group A, all the samples were negative for HBV with both assays. A comparison between the two assays was performed from samples in group B and C. Spearman's coefficient showed a significant correlation between the two assays (r = 0.948, P < 0.0001, Figure 1). The differences between viral loads obtained with the two assays versus the average for each specimen was analyzed with the Bland-Altman method[8] (Figure 2). Of the 158 samples, two (1.3%) samples had viral loads slightly above the upper limit and one (0.6%) sample was below the lower limit of the Fosun real-time PCR. However, viral loads of 75 (47.5%) samples were above the upper limit of the Amplicor test. In addition, HBV genotypes did not influence the quantitative results obtained with both assays.

Assay time and costs

The assay times and costs of both tests were compared. For a 24-sample work unit (controls were handled in the same way as clinical samples), Fosun real-time PCR required 3 h and the Amplicor test required 7 h. The cost of the Fosun real-time PCR per test, including consumables and DNA extraction reagents, was 5 US dollars. The cost of the Amplicor test was approximately 100 US dollars per test. If the samples were diluted and retested, the cost would be much higher.

DISCUSSION

China has the greatest burden of HBV infection in the world. Many commercially available HBV DNA assays in the United States and Europe are unaffordable for Chinese patients. Reliable but inexpensive HBV DNA assays are very important for control and treatment of HBV infection in low-income countries such as China. Fosun real-time PCR HBV DNA kits are produced by a Chinese company and approved by the Chinese SFDA for *in vitro* diagnostic use. Compared to the COBAS Amplicor test, this assay has equivalent reliability at a reduced cost.

Many studies have shown that real-time PCR is accurate, reproducible, sensitive, and rapid for quantitative detection of HBV levels in clinical samples[13,14,17-21]. Real-time PCR also has the widest dynamic range among all

the assays available commercially[14]. In this study, Fosun real-time PCR was able to quantitatively detect HBV in all seven reference sera from NICPBP, which showed a dynamic range of 8 logs. The majority (97.5%) of the HBV-positive samples used in this study were quantified within the manufacturer's recommended dynamic range for the Fosun real-time PCR, which indicates that this assay is superior to the Amplicor test for detection of HBV DNA in patients with high viral loads.

The results for the 158 clinical samples obtained with the Fosun real-time PCR and Amplicor tests were concordant very well. Comparison of the two assays for quantitative detection of HBV DNA levels in the 118 serum samples showed that the two sets of results were highly correlated ($r = 0.948$, $P < 0.001$). This was in accordance with the results of previous studies that have evaluated other real-time PCR assays for quantitation of HBV DNA[13,14,22-26].

One of the advantages of the Fosun real-time PCR kits is its low cost compared to that of the Amplicor test (5 *versus* 100 US dollars per test) and other commercially available tests[14]. With its low cost, it can be used to monitor antiviral therapy in economically disadvantaged patients with chronic HBV infection. As recommended by the American Association for Study of Liver Diseases and the Asian-Pacific Consensus Update Working Party on Chronic Hepatitis B, HBV DNA should be monitored at least every 3 mo during therapy. After the end of therapy, HBV DNA should be monitored monthly for the first 3 mo and then every 3-6 mo[27,28]. This will be a great economic burden for chronic hepatitis B patients in countries such as China, where most people have no medical insurance. Reliable and inexpensive assays for quantitation of HBV DNA are helpful for controlling HBV infection in such countries.

As with many other real-time PCR tests, the Fosun real-time PCR has no internal control included in the test. Internal controls can compensate for the differences in DNA extraction efficiency between specimens, and possible PCR inhibition in the reaction mixture[14,29-31]. In addition, standardization with international reference standards is also needed for wide acceptance of this assay. Further comparison with other commercially available assays such as the COBAS TaqMan 48 real-time PCR system will be helpful for evaluating the clinical performance of the Fosun real-time PCR assay.

In conclusion, the Fosun real-time PCR HBV DNA kit, with its much lower costs, is capable of providing reliable and rapid HBV DNA quantitation, which is useful for monitoring HBV DNA levels in patients with chronic hepatitis B.

COMMENTS

Background

Commercial real-time PCR assays are widely used for quantitation of hepatitis B virus (HBV) DNA in China. However, these assays have not been fully evaluated for their clinical performance.

Research frontier

Several methods are routinely used for quantitation of HBV DNA in serum or plasma samples. However, differences do exist among these methods. Comparison and evaluation of these methods are the focus of research efforts.

Related publications

Lindh *et al* and Gordillo *et al* have evaluated the clinical performance of commercial real-time PCR assays. However, they have not compared the cost and time between the real-time PCR and reference methods.

Innovations and breakthroughs

This study compared a real-time PCR assay widely used in China with the COBAS Amplicor test, which is widely used in the United States and Europe, for detection of HBV DNA in serum samples. Clinical performance, time required, and cost of the assays were analyzed.

Applications

This commercial PCR assay is suitable for quantitation of HBV DNA in serum samples.

Terminology

TaqMan PCR is one of the real-time PCR methods for the determination of copy number of PCR templates. It requires a pair of PCR primers and a fluorogenic probe, which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. The fluorescence can be monitored at frequent intervals during the PCR reaction with a real-time PCR machine.

Peer review

This is a well designed and prepared study with important conclusions. The most important point is that the real-time PCR is significantly cheaper than other commercial tests.

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CASE REPORT

Secondary aortoduodenal fistula

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Abstract

Aorto-duodenal fistulae (ADF) are the most frequent aorto-enteric fistulae (80%), presenting with upper gastrointestinal bleeding. We report the first case of a man with a secondary aorto-duodenal fistula presenting with a history of persistent occlusive syndrome. A 59-year old man who underwent an aortic-bi-femoral bypass 5 years ago, presented with dyspepsia and biliary vomiting. Computed tomography scan showed in the third duodenal segment the presence of inflammatory tissue with air bubbles between the duodenum and prosthesis, adherent to the duodenum. The patient was submitted to surgery, during which the prosthesis was detached from the duodenum, the intestine failed to close and a gastro-jejunal anastomosis was performed. The post-operative course was simple, secondary ADF was a complication (0.3%-2%) of aortic surgery. Mechanical erosion of the prosthetic material into the bowel was due to the lack of interposed retroperitoneal tissue or the excessive pulsation of redundantly placed grafts or septic procedures. The third or fourth duodenal segment was most frequently involved. Diagnosis of ADF was difficult. Surgical treatment is always recommended by explorative laparotomy. ADF must be suspected whenever a patient with aortic prosthesis has digestive bleeding or unexplained obstructive syndrome. Rarely the clinical picture of ADF is subtle presenting as an obstructive syndrome and in these cases the principal goal is to effectively relieve the mechanical bowel obstruction.

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Key words: Aorto-duodenal fistula; Surgery; Dyspepsia; Duodenotomy; Explorative laparotomy

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INTRODUCTION

Aorto-duodenal fistulae (ADF) are the most frequent aorto-enteric fistulae (80%). The most frequent presenting sign of ADF is upper gastrointestinal bleeding (UGI). We report the first case of a man with a secondary aortoduodenal fistula presenting with a history of persistent occlusive syndrome, which was treated with surgical exploration.

CASE REPORT

A 59-year old male patient who underwent an aortic-bi-femoral bypass 5 years ago, was admitted to the Emergency Room for a 5-day history of persistent occlusive syndrome with dyspepsia and biliary vomiting. Physical examination revealed a distended abdominal pain. Full blood examination was unremarkable.

Computed tomography (CT) scan showed in the third duodenal segment the presence of an area with the characteristics of inflammatory tissue including air bubbles between the duodenum and aortic-bi-femoral prosthesis adherent to the third duodenal portion ("comma sign") (Figure 1). Microbiological cultures and scintigraphy were unremarkable. Esophago-gastro-duodenoscopy showed the aortic prosthesis crossing the third segment of the duodenal wall occluding the intestinal lumen (Figure 2). At laparotomy, after viscerolisis, the prosthesis was detached from the duodenal wall and the intestine failed to close transversely (Figure 3). To protect the intestinal wall, a pedunculated fragment of the greater omentum was placed between the duodenum and aortic bypass. Furthermore, a gastrojejunal Roux anastomosis was employed. The prosthesis was not changed because there were no local or systemic signs of infection. The post-operative course was uneventful. The patient was discharged 10 days after operation and received a regular follow-up for 6 mo.

DISCUSSION

ADF are the most frequent aorto-enteric fistulae (80%)[1]. They may be primary due to a spontaneous communication between the lumen of aortic aneurysm

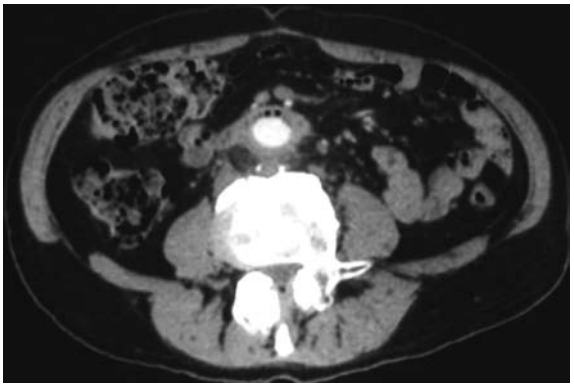


Figure 1 CT scan showing an area with the characteristics of inflammatory tissue including air bubbles between the duodenum and aortic-bi-femoral prosthesis adherent to the third duodenal portion.

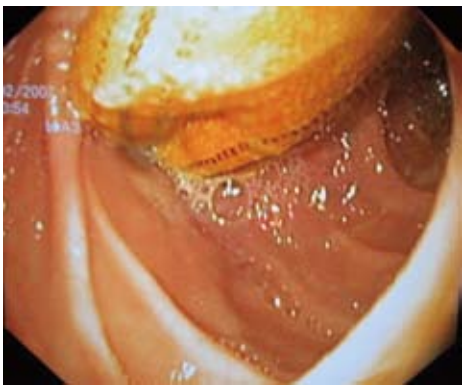


Figure 2 Esophago-gastro-duodenoscopy showing the aortic prosthesis crossing the third segment of the duodenal wall occluding the intestinal lumen.

and intestinal loop, or secondary due to surgical repair of aneurysms with prosthetic implants. The most frequent presenting sign of ADF is UGI bleeding. Clinical suspicion is essential in the diagnosis of ADF and the most commonly used techniques for its diagnosis are esophago-gastro-duodenoscopy (EGDS) and CT.

Secondary ADF is an uncommon (0.3%-2%) and life-threatening long-term complication of aortic reconstructive surgery[2]. Although the exact pathogenesis remains speculative, mechanical erosion of the prosthetic material into the adjacent bowel may be due to the lack of interposed retroperitoneal tissue or the excessive pulsation of redundantly placed grafts or septic procedures (low-grade, Staphylococcus epidermidis "biofilm" infection). Finally, some authors have described cases of inadequate prosthetic materials[2,3]. In our case, ADF formation was related to graft pulsation on the duodenal wall (in this patient, the blood pressure was stably high and not controlled by drugs).

The time between the first intervention and development of fistula can vary from months to years[3,4]. The presentation is often subtle with herald bleeding followed by a period of grace, or catastrophic bleeding, or rarely an episode of intestinal obstruction. The third or fourth duodenal segment is the most frequently involved site. In Dacron prosthesis patients, fistula develops in the

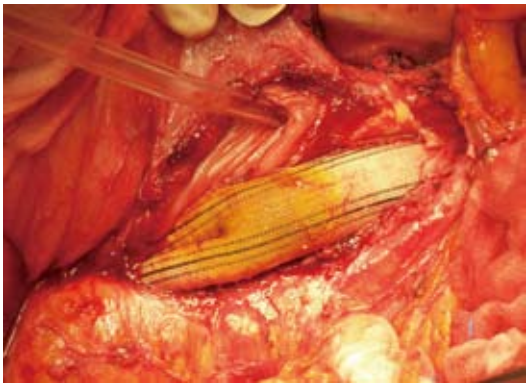


Figure 3 Isolated prosthesis in the context of eroded duodenal wall during laparotomy (the tube indicates the proximal duodenal lumen).

proximal graft tract opening in the third segment of the duodenum[1]. The right diagnosis of ADF can be difficult. It needs an early and correct suspicion index to establish the diagnosis of ADF in all patients who have undergone aortic graft surgery and present with gastrointestinal haemorrhage, sometimes fever, abdominal pain or mass or obstructive syndrome[5,6].

Though difficult, diagnosis can be achieved through EGDS or CT scan. Additional diagnostic procedures are often not useful. EGDS, the main diagnostic procedure, is able to demonstrate the fistula and rule out other possible causes of obstruction, but the most important tool to achieve diagnosis is clinical suspicion[3,6]. Because of the high mortality and morbidity, associated with secondary aorto-enteric fistula, surgical treatment is always recommended. Explorative laparotomy is the treatment of choice. Surgical procedures help guide the diagnosis and constitute the main part of treatment with suture of the duodenum and evaluation of vascular prosthesis.

In the case of non-treated aortic-enteric fistula presenting with massive UGI-bleeding, the mortality rate is near to 100%. While the morbidity (limb loss in 10%-40%) and mortality related to treated ADF are also high (75%) and require preventive measures, including more particularly delicate surgery and antibiotic therapy in case of any episode of infection. Several surgical procedures are possible[4,7].

ADF must be suspected whenever a patient with aortic prosthesis has digestive bleeding or unexplained obstructive syndrome[8]. More rarely, the clinical picture of ADF is subtle presenting as an obstructive syndrome and in these cases the principal goal is to effectively relieve the mechanical bowel obstruction. Further study is necessary to establish the more effective diagnostic mode.

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Natural killer T cells and non-alcoholic fatty liver disease: Fat chews on the immune system

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Abstract

Natural killer T cells (NKT) are an important subset of T lymphocytes. They are unique in their ability to produce both T helper 1 and T helper 2 associated cytokines, thus being capable of steering the immune system into either inflammation or tolerance. Disruption of NKT cell numbers or function results in severe deficits in immune surveillance against pathogens and tumor cells. Growing experimental evidence suggests that hepatosteatosis may reduce resident hepatic as well as peripheral NKT cells. Those models of hepatosteatosis and the change in NKT cell numbers are associated with a disruption of cytokine homeostasis, resulting in a more pronounced release of proinflammatory cytokines which renders the steatotic liver highly susceptible to secondary insults. In this letter to the editor, we focus on recently published data in the *World Journal of Gastroenterology* by Xu and colleagues demonstrating reduced peripheral NKT cells in patients with non-alcoholic fatty liver disease, compare those findings with ours and others in different animal models of hepatosteatosis, and hypothesize about the potential underlying mechanism.

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Key words: Obesity; Hepatosteatosis; Metabolic syndrome

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TO THE EDITOR

We are excited to read a recent report in the *World Journal of Gastroenterology* by Xu and colleagues demonstrating a reduction in the numbers of peripheral natural killer T cell (NKT) cells in patients with non-alcoholic fatty liver disease[1]. A growing body of experimental data demonstrates a link between hepatosteatosis and defects in NKT numbers and/or function. Over the past years, reductions in NKT cell numbers was reported in the fatty liver by a number of groups including ours[2-4]. Observations by our group show an inverse correlation between hepatic NKT cell numbers with the accumulation of hepatic lipid using the choline-deficient diet model of hepatosteatosis[4]. Although different animal models of diet-induced hepatic steatosis using high fat, high sucrose and choline-deficient diet or leptin deficient *ob/ob* mice showed depletion or decrease in hepatic NKT cell numbers[2-4], it has remained unclear if those findings were relevant to human fatty liver disease.

NKT cells play a critical role both in the liver and peripherally in the regulation of the innate and adaptive immune response. Decreased hepatic NKT cell numbers correlate with increased local production of pro-inflammatory T helper 1-associated cytokines[4,5]. We and others have shown that T helper 1-associated cytokines, such as tumor necrosis factor alpha, interleukin 12 and interferon gamma, are significantly elevated in steatotic livers in mouse models of obesity[3-5]. Similar findings have been reported in obese individuals with low levels of adiponectin and increased levels of circulating tumor necrosis factor alpha[6,7], although those studies did not examine hepatic or peripheral NKT cell numbers. NKT cells are unique in their ability to produce both T helper 1- and T helper 2-associated cytokines. The loss of NKT cells likely contributes to disrupted cytokine balance within the liver[5,8,9]. Consistent with their function as a regulator of the immune response, loss of NKT cell-associated interleukin 4 production promotes beta cell destruction in the *db/db* mouse model of type I diabetes[10]. Finally, NKT cells also function to clear tumor cells within the liver, as NKT cell activation promotes tumor cell clearance in several mouse models of hepatic metastasis[11]. Therefore, it is apparent that NKT cells play an important immunological and potentially regulatory role in both the liver and peripheral organs. The ability of hepatic lipid accumulation to alter this multifunctional cell population may have important and widespread pathophysiological consequences.

It remains unclear how hepatosteatosis reduces hepatic

and peripheral NKT cells. Previous studies implicated that interleukin 12 is a key activator of NKT cells, a process which is linked to activation-induced cell death of this cell population[12]. Therefore, it is likely that increased levels of interleukin 12 in fatty livers directly reduce the viability of NKT cells. We hypothesize that increased interleukin 12 expression in combination with hepatic lipid accumulation might be directly responsible for the reduced NKT cell population in non-alcoholic fatty liver disease (NAFLD).

The current study by Xu and colleagues is the first of its kind to establish this inverse relationship between NKT cell numbers and hepatosteatosis in humans and provides strong support to animal models of steatotic liver disease and immune cell dysfunction. Future research should focus on hepatic resident NKT cell numbers in humans suffering from NAFLD, and the relationship between hepatic and systemic cytokine levels of those patients should be monitored.

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Present 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 γ (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.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyr*A, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
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[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

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EDITORIAL

Management of biliary complications after orthotopic liver transplantation: The role of endoscopy

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Abstract

Biliary complications are significant causes of morbidity and mortality after orthotopic liver transplantation (OLT). The estimated incidence of biliary complications after OLT ranges between 10%-25%, however, these numbers continue to decline due to improvement in surgical techniques. The most common biliary complications are strictures (both anastomotic and non-anastomotic) and bile leaks. Most of these problems can be appropriately managed with endoscopic retrograde cholangiography (ERC). Other complications such as bile duct stones, bile casts, sphincter of Oddi dysfunction, and hemobilia, are less frequent and also can be managed with ERC. This article will review the risk factors, diagnosis, and endoscopic management of the most common biliary complications after OLT.

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Key words: Encoscopic retrograde cholangiopancreatography; Orthotopic liver transplantation; Biliary strictures; Bile leaks

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INTRODUCTION

Complications of the biliary tract after orthotopic liver transplantation (OLT) are an important cause of morbidity and mortality in liver transplant recipients. The most frequent biliary complications following LT are biliary tract strictures, bile leaks, and bile duct stones. Biliary complications arising after a liver transplantation occur in 10% to 25% of cases, with biliary strictures, bile leaks, and bile duct stones accounting for the majority of the complications[1-6].

These complications can be successfully managed with endoscopic retrograde cholangiography (ERC) in most cases. This article will review the different types of biliary complications after OLT and their endoscopic management.

RISK FACTORS

Risk factors for the development of biliary complications after OLT are shown in Table 1. A major risk factor is the type of biliary reconstruction (duct-to-duct choledocho-choledochostomy *vs* Roux-en-Y choledochojejunostomy). The risk of biliary complications with the Roux en Y reconstruction is similar or only slightly higher compared with the duct-to-duct anastomosis[2,7-10]. Duct-to-duct anastomosis of the common bile duct has the advantage of including easy access to the biliary system after OLT and preservation of the sphincter of Oddi[11]. The use of T-tubes has also been implicated as a risk factor after OLT, although this issue is controversial. Some centers prefer the routine use of T-tubes for bridging of the biliary anastomosis as it allows the assessment of the quality and quantity of bile, biliary tract anatomy, and reduces the incidence of strictures. Despite these benefits, comparative studies indicate that routine T-tube placement is associated with higher incidence of bile leaks and cholangitis[12-15].

Acute hepatic artery thrombosis, which may lead to ischemic strictures unless vascular flow is immediately reconstituted, is one of the major risk factors for the development of complications after OLT[12]. Other risk factors for the development of biliary complications include technical factors during surgery (excess dissection of periductal tissue during procurement and tension of the duct anastomosis), ischemia/reperfusion injury, cytomegalovirus infection, non-heart beating donors, ABO incompatibility and primary sclerosing cholangitis[12,16-22].

DIAGNOSIS

Patients may present with asymptomatic elevations of serum transaminases, serum bilirubin, alkaline phosphatase and/or γ-glutamyl transferase levels. In some cases patients may have non-specific symptoms, such as fever and anorexia[10,11,16]. In the initial evaluation of these

Table 1 Risk factors for the development of biliary complications after OLT

1	T-tube
2	Roux-en-Y anastomosis
3	Ischemia/Reperfusion injury
4	Acute hepatic artery thrombosis
5	Infections
6	ABO mismatch
7	Non-heart beating donors
8	Primary sclerosis cholangitis

patients, an abdominal/liver ultrasound (US) should be performed to evaluate the biliary tree and the hepatic vessels. If hepatic artery stenosis is suspected, a hepatic angiography is usually indicated. Liver biopsy is useful in order to exclude rejection, but histological features of biliary obstruction can sometimes be misleading and confused with rejection or hepatitis C recurrence[23]. In cases where there is biliary dilation on the abdominal US, liver biopsy is not initially performed due to the risk of causing a bile leak. The yield of abdominal US in detecting biliary complications is sometimes limited by a lack of good sensitivity and accuracy to detect common bile duct obstruction or stones. Hence, the absence of bile duct dilation on US should not preclude further evaluation with other sensitive techniques (see below) in patients in whom there is suspicion of biliary tract complications.

If there is a strong clinical suspicion of bile duct obstruction or bile leak, a cholangiogram, by means of endoscopy or radiology, should always be performed. In recent years, the role of magnetic resonance cholangiopancreatography (MRCP) as a tool to evaluate the biliary tract of post-OLT patients has significantly improved. Recent studies demonstrate that MRCP has high sensitivity and specificity in detecting biliary complications after OLT (93%-100% and 92%-98%, respectively). MRCP is particularly useful in patients with a low suspicion of biliary complications or when there is a low probability of ERC or percutaneous transhepatic cholangiography (PTC)[24,25] (Figure 1). In patients with an indwelling T-tube, a T-tube cholangiography is the method of choice to evaluate the biliary tract. The best diagnostic approach (ERC *vs* PTC) depends on the type of biliary reconstruction, the likelihood of therapeutic intervention, and the center's expertise. ERC is probably the best diagnostic intervention in patients with duct-to-duct anastomosis, while PTC should be performed in cases of failed ERC or in patients with a Roux-en-Y anastomosis. In high volume centers with experienced endoscopists, ERC can be successfully performed with a variable stiffness pediatric colonoscope. In one report from the Mayo Clinic, 22 out of 31 patients post-OLT with Roux-en-Y anastomosis referred for ERC were successfully treated with endoscopy[26].

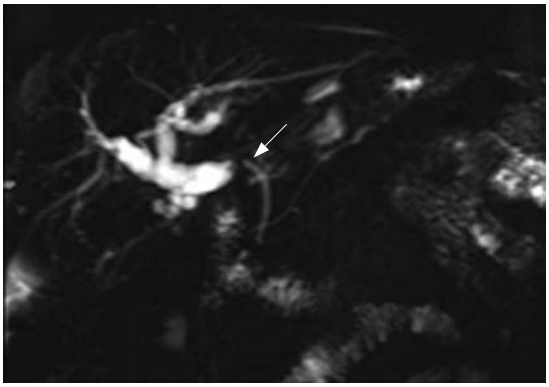


Figure 1 Magnetic resonance cholagiopancreatography (MRCP) image of the biliary tract of a patient that developed abnormal liver enzymes 8 mo after liver transplantation. The MRCP showed a severe biliary stricture at the anastomotic site (arrow).

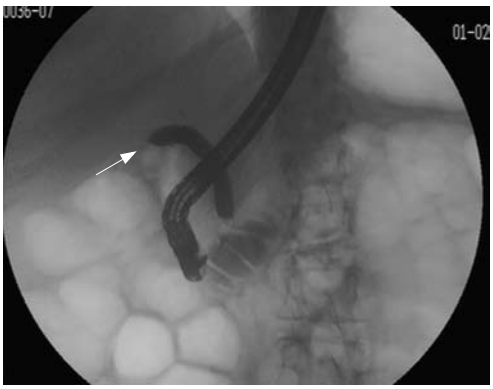


Figure 2 Endoscopic retrograde cholangiography (ERC) in a patient that developed pruritus and cholestasis 6 mo after liver transplantation. The ERC showed a severe anastomosis stricture (arrow). This stricture was successfully managed with biliary balloon dilation and stent placement.

MANAGEMENT

Strictures

Bile duct strictures are the most common biliary complication after OLT with an incidence ranging between 4% and 16%[1-6,10,11,16,27-30]. Strictures are divided into early (within 1 mo of OLT, usually related to technical problems) and late strictures (after 1 mo of OLT, mainly secondary to vascular insufficiency). In addition, bile duct strictures are classified as anastomotic (AS) or non-anastomotic (NAS) depending on the stricture site[16].

In some patients, within the first two months after surgery, a narrowing of the anastomosis (mainly due to postoperative edema and inflammation) may occur and in general responds to one session of endoscopic biliary balloon dilation (6-8 mm) and plastic stent placement (7 French) with removal 6-8 wk later[10]. The majority of true AS occur between the first 3 and 12 mo after OLT (Figure 2). The preferred endoscopic treatment for AS is the repeated combination of biliary balloon dilation (6-10 mm) plus the use of plastic stents ranging from 7-11.5 F with increasing diameter and number, if possible[30-33]. These strictures often recur (recurrence rate of 30%-40%) and in many cases require long-term stenting (up to 24 mo)[31]. The efficacy of this combined approach has been confirmed in at least two studies. In one study of 24 patients, combination treatment of biliary balloon dilatation and stent placement was more effective than balloon dilation alone (success rate of 88% *vs* 37.5%, respectively)[32]. In another study, 22 patients with AS

Table 2 Key points in the management of bile duct strictures

- Bile duct strictures can be classified as anastomotic or non-anastomotic
- Early anastomotic strictures usually respond to endoscopic dilation and short-term stenting
- Late anastomotic strictures have a high rate of recurrence (30%-40%) requiring long-term stenting (up to 24 mo).
- If endoscopic or percutaneous treatment fails, surgical repair or conversion to Roux-en-Y choledochojejunostomy may be necessary
- Non-anastomotic strictures require long-term stenting
- Some patients do not respond and finally undergo re-transplantation or die because of this complication

underwent endoscopic dilation plus maximal stenting [up to 4 stents (range 7-11.5 French)] and in 81% cases complete resolution of the stenosis was achieved in a mean period of 4.6 mo[33]. It is recommended that plastic stents be exchanged every 3-mo to avoid stent occlusion. With this approach 67%-91% of patients respond to endoscopic treatment alone[3,29,30,32,33]. In patients with Roux-en-Y anastomosis, PTC and dilation followed by placement of a percutaneous transhepatic catheter are recommended. If endoscopic or percutaneous therapy fails, surgical repair or conversion to a Roux-en-Y anastomosis may be necessary for successful long-term outcome.

NAS are mainly due to vascular problems and ischemia. Recurrence of primary sclerosing cholangitis is also a cause of NAS. The incidence of NAS ranges between 0.5% and 9.6%[2,6,8,28,29,34,35]. These strictures tend to occur earlier than AS (3-6 mo after OLT)[29,34]. Endoscopic treatment of NAS is similar to that for AS, and typically consists of 4-6 mm biliary balloon dilation (compared to 6-10 mm for AS) and placement of plastic stents[29]. The time to response is longer in NAS compared to AS. In one study, the median time to response was 185 d for NAS compared to 67 d for AS[35]. Despite endoscopic treatment with biliary balloon dilation and stenting, nearly 30%-50% of patients undergo re-transplantation or have a fatal outcome as a consequence of NAS[11,16,29,34,36]. The key features in the management of bile duct strictures post-OLT are described in Table 2.

Bile leaks

Bile leaks are a common complication after OLT with an incidence ranging between 2%-25%[1-6,16,27,37]. Leakage may arise from the anastomosis, the cystic duct, or T-tube (Figure 3). Bile leaks can also be divided into early (within 4 wk after transplantation) and late[16]. Early bile leaks usually arise from the anastomosis and are related to technical problems, such as insufficient blood flow from the hepatic artery to the anastomosis and other factors described in Table 1. Patients with bile leaks may be asymptomatic (bile leak detected by the presence of fluid collection on abdominal ultrasound or CT scan) or present with a clinical picture of peritonitis. In patients with a T-tube in place, bile leaks may be detected with a T-tube cholangiogram and managed by leaving the tube open without further intervention[16]. In patients without a T-tube, ERC is the preferred diagnostic test. In addition, ERC with biliary sphincterotomy and stent placement is the method of choice to treat bile leaks (success rate of 95%)[4,5,38,39]. In these patients, the stent is left in place for 2-3 mo[16]. Small leaks can be managed with biliary sphincterotomy alone[40]. In patients with Roux-en-Y anastomosis, bile leaks can be managed with percutaneous internal-external drainage, however, they often require surgical management. Late bile leaks are commonly related to the removal of the T-tube because the tract is often not mature due to immunosuppressive therapy. Patients that complain of abdominal pain after T-tube removal should raise the concern for a bile leak. In these cases, symptoms resolve with analgesia without further intervention[1-6,16,37,41].



Figure 3 Endoscopic retrograde cholangiography (ERC) image of a biliary leak in a patient after a liver transplantation. The arrow is pointing to the area of extravasation of contrast from the biliary tree.

OTHER COMPLICATIONS

Biloma

Bilomas occur due to bile rupture and extravasation of bile within the liver or the abdominal cavity. If the biloma communicates with the biliary tree, it may resolve spontaneously or occasionally with the placement of a plastic stent in the extrahepatic bile duct. Large bilomas not communicating with the bile ducts should be treated with percutaneous drainage along with antibiotics. If bile leaks cannot be effectively controlled with the abovementioned treatments, surgery is indicated.

Common bile duct filling defects

Most filling defects are caused by gallstones[5]. Other causes include sludge, blood clots, casts and migrated stents. Biliary stones and sludge are relatively common after OLT, with an incidence between 3% and 12%[3-5,8,42]. Factors related to the formation of stones and sludge are the presence of strictures, bacterial infection and obstruction[10,16,27]. In many cases (66%), one session of ERC with sphincterotomy stone extraction is adequate to clear the duct[3]. Bile casts are seen in the setting of ischemia. Clearance of casts may be difficult to achieve with endoscopic methods. Various combinations of sphincterotomy, balloon and basket extraction, stent placement and lithotripsy are often necessary and many of these patients require management with PTC[43].

Sphincter of Oddi dysfunction

It has been postulated that denervation of the common bile duct in the ampullary region secondary to surgical

intervention may lead to the development of a hypertonic sphincter causing sphincter of Oddi dysfunction[11,27]. Endoscopy therapy with biliary sphincterotomy is the treatment of choice in cases of a uniformly dilated common bile duct with cholestasis without any other obvious cause for abnormal liver chemistries.

Mucocele

A mucocele is a rare complication after OLT caused by a collection of mucus from the cells lining the cystic duct remnant. The diagnosis can be confirmed with MRCP. Patients usually require surgical or radiological drainage[16,44].

CONCLUSION

Biliary complications arising after OLT occur in approximately 10%-25% of cases. Biliary strictures, bile leaks and bile duct stones account for the majority of these complications. The diagnostic approach of biliary complications after OLT should begin with a liver US and Doppler examination of the hepatic artery. In cases with a strong clinical and radiological suspicion of biliary pathology, the patient should proceed directly to ERC. MRCP is an excellent diagnostic tool for the evaluation of the biliary tree after OLT and should be performed in cases where there is a lower suspicion for biliary pathology. Patients with AS and NAS should undergo repeated ERC sessions with biliary balloon dilatation and placement of plastic stents to achieve maximal diameter with routine stent changes every 3 mo. In most cases, patients require between 3 and 5 sessions. In patients with bile leaks, ERC with or without sphincterotomy with placement of a plastic stent, which should be left in place for 2-3 mo, is recommended. Other complications such as common bile duct stones, casts, mucocele or sphincter of Oddi dysfunction can be managed successfully with ERC and standard techniques for removal of stones and sphincterotomy.

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REVIEW

Osteoporosis in celiac disease and in endocrine and reproductive disorders

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Abstract

As the increase in lifespan brings to light diseases that were previously not clinically detectable, osteoporosis has become an issue of worldwide significance. The disease is marked by a loss of bone mass; the bones become less dense, fragile and more prone to fracturing. Because it is regulated by endocrine and environmental factors, osteoporosis presents a multifactorial etiopathogenesis, with the genetic component accounting for 70% of an individual variation in bone mass density (BMD), the principal determinant, with age, of fracture risk. Pathological conditions such as celiac disease (CD) exacerbate the process of bone loss, so that the occurrence of osteoporosis in celiac subjects is of particular note: indeed, the screening of osteoporosis patients for this disease is advisable, since it may be the only sign of undiagnosed CD. An increase in interleukin IL-1β, of the IL-1 system, in the relatives of celiac patients confirms the genetic predisposition to osteoporosis and its presence is evidence of an association between the two conditions. The direct effect on the bones of CD is secondary to poor absorption of calcium and vitamin D. In women osteoporosis is indirectly associated with early menopause and amenorrhea, and it may follow prolonged breast-feeding and frequent pregnancies, while in men it is associated with hypogonadism and GH deficit. These endocrine and non-endocrine factors exert their effects on bones by modulating the RANK/RANK-L/OPG system. An appropriate lifestyle from adolescence onwards, together with early diagnosis of and treatment for CD and primary and secondary endocrine pathologies are important for the prevention of damage to the bones.

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Key words: Osteoporosis; Celiac disease; Menopause; Estrogens; Hypogonadism; Micronutrients

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INTRODUCTION

Osteoporosis has only recently been accorded the clinical importance that its evolution and potential health impact deserve. Thanks to considerable increases in life expectancy this pathology is becoming more visible, and is now treated as a serious public health issue, as well as one of social and economic importance.

Osteoporosis generally manifests in late middle age, but its causes are rooted in the years of development[1]: a lower peak of bone mass in the first 20 years of life can influence the evolution of this disease in later years. The impoverishment of bone mass may become irreversible and lead to serious disability necessitating long-term assistance and treatment, with increasing social and economic costs: for these reasons prevention is of fundamental importance. Prevention should be initiated early on, particularly during adolescence, when calcium ingested with food is absorbed by the organism and contributes effectively to consolidating bone density; it is also important that children and young people engage in regular physical activities from the earliest schooldays through to the completion of secondary schooling. In diseases such as osteoporosis an individual's gender, race and constitution, as well as variations in complex endocrine systems associated with factors such as the menopause, aging and the presence of other pathologies,

can all interact with the lifestyle to determine its onset[2].

In order better to understand the changes in endocrine-environmental factors that can cause damage to bone tissue, the following brief description of its physiology and pathology may be helpful.

THE PHYSIOPATHOLOGY OF OSTEOPOROSIS

Bone is a mineralized tissue consisting of an organic matrix of collagenous fibers (proteins) dispersed throughout an inorganic mass of minerals (calcium hydroxyapatite). Osteoporosis is a quantitative and qualitative alteration in the components of this tissue, in which the process of demineralization becomes intense and prolonged and minerals are used up more quickly than they can be replaced, to the point where bones become fragile and break easily. In this it differs from osteomalacia, which is marked by qualitative alterations due to mineralization defects of the proteinic matrix.

The organic and inorganic matrix, together with the cells (osteoblasts, osteoclasts, osteocytes, lining cells) make the biodynamic units of the process of remodelling and resorption, the basic multicellular units (BMU).

The particular characteristic of bone tissue is that during an individual's lifetime it is subject to processes of transformation that can be summarized in the following stages:

- -the stage of bone mass increase that parallels growth in stature;
- -the stage of consolidation marked by a slow but steady increase in bone mass, which lasts until 20-25 years of age;
- -the stage at which each individual's peak bone mass is reached and in which the difference between men and women is evident;
- -the stage of decline in which bone mass is reduced and which lasts for the rest of an individual's life.

Thus, notwithstanding their apparent solidity, bones are the dynamic and continually evolving sites of two constant and inter-related processes (bone turnover): a slower process of accumulation and a more rapid one of resorption. Then there are the various functions in which calcium is involved: its endocrine function, nerve conduction, muscle contraction, cell metabolism and blood coagulation. Calcium is carried by the blood to and from all bodily tissues, where a dynamic equilibrium is reached between the body's use of calcium and the need to sustain the calcium load. This equilibrium is finely regulated by various endocrine, environmental and genetic factors, responsible for the multifactorial origin of osteoporosis, in which the genetic component plays an important part[3]. Numerous studies performed in recent years to identify the genes and allelic variants involved in osteoporosis have shown its polygenic determinants, but the identity of genes involved has not yet been disclosed. It is estimated that genetic factors account for 70% of individual variations in bone mass density (BMD), the principal determinant, with age, of fracture risk, and that BMD accounts for 70% of bone solidity.

The elucidation of the role of the (IL)-1 interleukin system, localized on chromosome 2q14-21 and comprising IL-α, IL-1β and the IL-1 receptor antagonist (IL-1ra), has shown that IL-α and IL-1β are potent stimulators of bone resorption by inducing the proliferation and differentiation of both osteoclast precursors and mature active osteoclasts[4].

Studies on the allelic variants of the IL-1 system in postmenopausal women of different ethnic origins have revealed the existence of associations between BMD and the system's different genetic polymorphisms. Investigations on the relations between polymorphisms of the IL-1 system and BMD in 220 postmenopausal Korean women aged between 48 and 70 years showed that BMD in those carrying the A2 allele of the IL-1ra gene was significantly lower than that of those who did not carry the allele. Moreover, the A2 allele was more frequent in women with osteoporosis than in those who did not suffer from the disease. There is thus a relation between this allele and BMD; it presumes that polymorphism of the IL-1ra gene is an important genetic factor that affects the BMD of Korean women[4].

Other studies have identified regulators of bone remodelling, such as the Ciz gene (Castor-interacting zinc finger protein), which could also possible be used as therapeutic targets[5] particularly for the treatment of individuals also affected by CD. The existence of an association between the two pathologies was demonstrated by a study performed by Taranta *et al*[6], in which the authors showed the presence of a cytokine imbalance affecting bone metabolism in the sera of celiac patients and the direct effect of these sera on bone cell activity *in vitro*. They found an increase in IL-1β and in tumor necrosis factor alpha (TNF-α) in all patients, while patients who did not follow a gluten-free diet (GFD: only sure treatment for CD) also had increased IL-6 and IL-18 and an additional increase in TNF-α. These findings suggest that the loss of BMD could be caused partly by this imbalance, which directly affects osteoclastogenesis and osteoblast activity.

Besides cytokines, parathyroid hormone (PTH), estrogens, androgens, corticosteroids and vitamin D all affect bones by modulating the receptor activator of nuclear factor B/receptor activator of nuclear factor B-ligand/osteoprotegerin (RANK/RANK-L/OPG) system[7].

The RANK-L is the final mediator of the cytokine (IL-1, TNF, IL-6) and hormone (PTH) network. This soluble polypeptide is produced by osteoblasts and expressed on their cell membrane and binds with high affinity to its specific RANK receptor, present in hemopoietic osteoclast precursors of bone marrow and on the membrane of osteoclasts. OPG is a polypeptide of 380 amino acids that is produced in several cell types, including those of the immune system and osteoblastic stromal cells, and has been identified as the soluble biological RANK-L inhibitor able to contrast its osteoclastogenetic, pro-resorption and anti-apoptotic effects on mature osteoclasts[8]. OPG is thought to act as a 'decoy' receptor, competing with the specific RANK receptor to inhibit RANK-L/RANK binding[7].

The importance of the increase in IL-1β is supported by its presence in relatives of celiac patients, confirming

the genetic susceptibility for bone loss pathogenesis[9]. Other studies of families and twins have produced further evidence that genetic factors play a significant role in osteoporosis and that they affect not only BMD but also skeletal geometry and bone turnover, with an increased fracture risk[3].

In addition to pathologies such as, for example, Cushing's disease and prolactinomas, other factors can indirectly affect bone homeostasis. One of these is stress due to physical strain, which produces a cytokine and hormonal imbalance. Strenuous physical exercise leads to an imbalance in which the central nervous system and the hypothalamus-pituitary-suprarenal axis react by increasing catecholamines, cortisol and the activation of corticotropin-releasing hormone (CRH) neurons, with consequences for gonad function. IL-6, growth hormone (GH) and prolactine (PRL) are also activated. It is worth emphasizing that physical exercise in men not only increases muscle mass but also has an indirect effect on bone structure. In female athletes the so-called 'exercise-related reproductive dysfunction' may be present, the consequences of which may include not only osteoporosis but also amenorrhea, infertility, food-related disorders, coronary disease and "euthyroid sick" syndrome[10].

As we have already seen, the phase of decreasing body mass, which continues throughout the lifespan, is a physiological fact: there nonetheless exist risk situations which, by interacting with the balance of hormonal (e.g. estrogen deficiency), nutritional (e.g. calcium deficiency) and environmental (e.g. lifestyle) factors, accelerate bone loss and can harm the organism[11]. Because bones are highly susceptible to interference, it is very important to maintain this balance which, together with genetic equilibrium, helps to preserve bone health. Pathologies such as celiac disease (CD), which manifests when genetic and environmental factors interact, can affect this equilibrium and increase the risk that an individual will develop bone diseases such as osteoporosis and osteomalacia[12].

On account of both its frequency (one in four women and one in eight men over 50) and related complications, osteoporosis represents a serious health emergency of considerable economic importance; when it is associated with CD it may cause additional damage to bone health; indeed, screening for CD is recommended for patients with idiopathic osteoporosis[13,14]. If these patients also suffer from endocrine-reproductive disorders, such screening becomes even more advisable, since these disturbances are a significant risk factor for osteoporosis[2].

SOME ASPECTS OF THE ETIOPATHOGENESIS OF CELIAC DISEASE

While discussing the association between CD and osteoporosis in persons also affected by reproductive problems, it is worth emphasizing certain aspects of the etiology and pathogenesis of celiac disease.

For genetically predisposed individuals, CD is a permanent gluten intolerance, for which the only treatment currently available is lifelong adherence to GFD. The disease is a chronic enteropathy featuring with villous atrophy, crypt hyperplasia and lymphocytosis in which fundamental in inflammatory processes occurs in the proximal part of small intestine mucosa. The adaptive immune pathway is thought to provide the major immune response, but recent evidence has also indicated the involvement of the innate immune system[15]. Besides increased T lymphocytes, other cell types are also increased, including B lymphocytes, NK cells, neutrophils, eosinophils, macrophages and mastocytes. In particular, a chronic recruitment of activated neutrophils is present even in completely normalized remission of CD[16].

In the pathogenesis of CD exposure of the mucosa of the small intestine to gluten leads to hyperproduction of cytokines by T cells, triggering an inflammatory process that includes the release of IL-2, IL-6, IFN-γ and TNF-α and activation of B lymphocytes with production of specific antibodies and the activation of autoimmune mechanisms. IL-15, a mediator of the innate immune system, is thought to be of particular importance in damage to the mucosa and in the persistence of inflammatory processes[17].

CD used to be considered a rare disease typical of infancy, with generalized symptoms of poor absorption combined with chronic diarrhea and slow growth. It is now recognized that along with this classic form of the disease there are other clinical and subclinical forms that may manifest later and in which there may be no intestinal symptoms; these forms can be associated with symptoms affecting the liver, thyroid, skin, reproductive system and bones[18]. The effects of CD on these systems are mediated not only by gluten but also by the combination of other genetic and environmental factors, indicating that CD, like osteoporosis, has a multifactorial pathogenesis[19]. The poor absorption induced by CD leads to deficiencies of nutrients such as iron, folic acid and vitamin K that are essential for organogenesis, of fat-soluble vitamins important for spermatogenesis, and of vitamin D and calcium, essential for the maintenance of bone structure[18]. Osteoporosis deserves particular consideration as a non-intestinal sign of CD; indeed, bone metabolism disorders and a decrease in BMD may be the only signs of otherwise 'silent' CD[20]. In particular, studies have shown that osteoporosis is more serious in individuals also affected by CD[18] and is an important example of a non-intestinal sign of the disease, a factor that acquires additional importance if we consider that 1% of the total population suffers from CD[21]. Osteoporosis is more prevalent in celiac (3.4%) than in non-celiac (0.2%) subjects. In addition, severe cases of CD are accompanied by more severe osteoporosis[13], and celiac individuals who also suffer from reproductive disorders are affected even more severely[2].

In light of the available evidence, it is useful to see how endocrine and reproductive factors interfere in osteoporosis, with special focus on problems associated with CD.

OSTEOPOROSIS IN WOMEN

For women the risk of osteoporosis is linked primarily to a fall in the production of estrogens, the female

steroid hormones *par excellence*. In order to understand the importance of these hormones, it is useful to emphasize that, as well as influencing reproduction and bone integrity, they are major actors in numerous physiological processes: it is thus no surprise to find that they are implicated in the development and progression of several diseases ranging from various types of tumor (e.g. breast, ovaries, colon-rectum, prostate, endometrium) to neurovegetative and cardiovascular disorders, insulin resistance, lupus erythematosus, endometriosis and obesity. In several of these diseases estrogens act through estrogen receptors (ER), and our understanding of this mechanism forms the basis of a number of treatments[22].

After the menopause the decrease in estrogen is evident in the absence of regular modulation of cellular activity in tissue[2]. The biological effects, mediated through the interaction between estrogen and ERα and ERβ, are expressed as a form of protective action in which bone turnover is inhibited, with a consequent reduction in both resorption and the formation of new bone[23].

Estrogen deficiency promotes calcitonin-induced hypocalcemia and a secondary increase in PTH consistent with the effects of calcitonin in increased bone turnover. By promoting the interaction of calcitonin and PTH, estrogens thus regulate calcium homeostasis and bone turnover[24]. In addition, patients with resection of the small intestine also show low levels of 25-hydroxyvitamin D [25(OH)D] associated with increased PTH and decreased BMD values[25]. This is of particular interest when we consider that this part of the intestine is the target of CD, a factor that makes a common pathogenetic mechanism for the two diseases all the more plausible.

The RANK/RANK-L/OPG cytokine system is essential to bone turnover biology; metabolic bone diseases are related to alterations in this system[26]. Functional links between bone remodelling and the immune system in inflammatory processes are also mediated, in part, by the RANK/RANK-L/OPG cytokine system. In these processes, neutrophils play a crucial role; RANK-L is expressed by inflammatory and normal neutrophils, unlike OPG and RANK, which are expressed only by neutrophils exposed to an inflammatory environment. This suggests that neutrophils may contribute to bone remodelling at inflammatory sites where they are present in significantly large numbers[27]. Interestingly in both active and remission CD enhanced neutrophiles infiltration is observed[16]. The risk of osteoporosis is higher in women suffering from CD, which can act both indirectly and directly. The indirect effects of CD include early menopause[28] and amenorrhea[29], two pathologies that are themselves an osteoporosis risk[18]. An early menopause in female patients increases the risk of early development of osteoporosis. Amenorrhea is a major clinical consequence of increased levels of PRLs, which in turn promote disruptions in BMD[30]. In some women other factors such as long periods of breast-feeding and frequent pregnancies may represent a risk of early osteoporosis[31].

In these conditions, latent CD may become manifest or be reactivated in women who have followed GFD for some time, suggesting the existence of immune or hormonal changes peculiar to pregnancy and puerperium[32], such as increased PRL which, as already emphasized, bring changes in BMD[30].

Early menopause should be treated by the immediate adoption of measures to prevent and treat osteoporosis, such as dietary supplements of calcium and vitamin D, physical exercise, not smoking, moderating alcohol consumption and, where necessary, the administration of drugs and/or hormone replacement therapy (HRT). These strategies are necessary to prevent increased fracture risk, largely because most patients are not aware that they have osteoporosis until a fracture occurs[33]. It is also advisable, during critical periods such as pregnancy and breast-feeding, to take calcium and vitamin D supplements[34].

To complete the effects of hormones on bone balance it should be remembered that hyperthyroidism, evident in low serum levels of TSH, is accompanied by a decrease in bone mass[35]. Over treatment for postmenopausal hypothyroidism can lead to a reduction in normal TSH values (0.5-4.0 mU/L), which, by causing subclinical hyperthyroidism, can lead to osteoporosis. It is useful here to remember that thyroiditis may first appear as periods of metabolic hyperfunction leading to hyperthyroidism.

In adults osteoporosis associated with hyperthyroidism is traditionally viewed as a secondary consequence of altered thyroid function[36]. Some studies have provided evidence for a direct effect of TSH on skeletal remodeling mediated *via* the TSH receptor (TSH-R) found on osteoblast and osteoclast precursors. Even a 50% reduction in TSH-R expression produces serious osteoporosis together with focal osteosclerosis (localized bone formation). These studies define a role for TSH as a single molecular switch in the independent control of both bone formation and resorption. In particular, TSH inhibits osteoclast formation and survival by interacting with the RANK-L/RANK system and TNF-α[37].

Because of their endocrine-environmental implications, forms of thyroiditis often occur in combination with CD[38] and are among the pathologies most frequently associated with it. Bearing in mind that forms of thyroiditis can lead to spontaneous abortion, neonatal mortality, retarded fetal growth and congenital malformations[39] and that CD has an important impact on the reproductive system[18], it can be appreciated that the genetic and environmental factors associated with these diseases are correlated. A direct effect of CD is that the process of bone remodeling is increased in these patients in relation to calcium malabsorption, secondary hyperparathyroidism and vitamin D deficiency, with marked bone loss[40].

A further consideration in support of a direct effect of CD in the development of osteoporosis is that patients with this disorder suffer not only from generalized poor absorption but also from a deficiency of specific micronutrients fundamental both to normal male and female reproductive development[18] and to the regular growth and maintenance of bone tissue[12]. The presence of osteoporosis in CD patients, particularly in subclinical cases, thus underscores the significance of a shortage of specific nutritional elements. This is further emphasised by the malabsorption of calcium and vitamin D that affects women with CD. Eastell[41] highlights this fact given that women are affected not only by a lack of estrogens but also by a deficiency of these important

micronutrients in the pathogenesis of osteoporosis. Regarding the loss of calcium it is useful to emphasize that this occurs both as a direct consequence of damage to the mucosa[42] and with secondary steatorrhea, one of the major effects of CD, through a chelating mechanism involving the intraluminal fats[43,44]. All the above considerations assume even greater significance if we remember that, in general, the poor absorption of calcium by postmenopausal women and estrogen-related calcium deficiency unquestionably represent a major risk factor for postmenopausal osteoporosis, as evidenced by improved calcium levels following hormone replacement therapy (HRT). Approximately 20 million women worldwide use HRT, and while its beneficial effects for osteoporosis and menopausal symptoms are known, the well-documented higher risk of breast tumors in these subjects cannot justify its long-term use. Besides, several recent trials have indicated that HRT carries a higher risk of cardiac and cerebral events, contradicting its protective effects on the cardio-and cerebrovascular systems[45].

With regard to preventing or slowing the progression of chronic diseases such as osteoporosis and combating the increased risk of breast cancer, the consumption of soy isoflavones is gaining ground, particularly in the US. These natural substances are similar to estrogen in their action; their beneficial effects on bones may be specific to each phase of an individual's life, as well as depend on the number of estrogen receptors and on the endogenous hormonal environment. Menopausal or premenopausal women, who have greater numbers of these receptors, may be more receptive to the therapeutic effects of isoflavones on bone loss than postmenopausal women, in whom estrogen receptors decrease. Studies on pubertal laboratory animals exposed to high levels of soy isoflavones showed negative effects. This is important if we remember that no clinical studies on adolescents are available to confirm or challenge research carried out on animals. Studies should, in any case, be performed on individuals at all stages of the lifespan to identify possible long-term effects of these substances: indeed, this aspect should be the basis of research aimed at assessing the risk-benefit ratio[46]. Bearing in mind that osteoporosis is associated with genetic-environmental pathologies such as CD, the use of soy isoflavones, which albeit gluten-free nonetheless contain estrogen-like substances, should be carefully evaluated.

Alongside calcium and vitamin D, the prevention of osteoporosis through the development and maintenance of bone mass also calls for other vitamins (A, C, E, K) and minerals (phosphorus, fluoride, iron, zinc, copper, boron) that, by improving BMD, are necessary for a normal metabolism[47] and for bone turnover[48]. The administration of these nutrients from adolescence, particularly in individuals with CD, could help to prevent the onset of osteoporosis. Among them, vitamin K merits special attention, given that its deficit in pregnant women can harm the fetus, leading to chondrodysplasia punctata with nasal hypoplasia and spinal cord abnormalities[49]. In menopausal women vitamin K deficiency disrupts bone calcification on account of reduced osteocalcin (GLA) carboxylation[50]. A deficiency of zinc is also particularly important, as this mineral affects BMD through its effect on the ovaries and consequent changes in estrogen levels; moreover, low maternal serum zinc levels are associated with fetal malformations such as neural tube deficit[51]. This suggests that a shortage of these micronutrients may also have a harmful effect on fetal bone metabolism. Because of the direct involvement of CD in the availability of micronutrients, the above factors may be more significant in celiac women who do not follow the GFD correctly or who are unaware they have the disease.

OSTEOPOROSIS IN MEN

The longer lifespan of humans brings an increased risk of osteoporosis in men and in women, but while in women it is undoubtedly associated with the menopause, in men it is often linked to the effect of GH and hypogonadism[52]. During infancy and adolescence GH stimulates growth and sexual development as well as increasing muscle mass and the formation of bone tissue, while in adults it is important for metabolism in various tissues. The association between CD and GH deficiency is evident in the low stature of celiac children, although after a year on GFD these children will resume normal growth, particularly if the diet is combined with hormone therapy[53,54].

With regard to hypogonadism, osteoporosis is strongly influenced by the homeostasis of steroid hormones[55], changes in which are indicated as one of the commonest causes of this type of pathology[56], while the presence of CD may represent an additional risk factor for osteoporosis[57]. Celiac males are at greater risk of infertility and hypogonadism: indeed, the GFD can improve both sperm count and sperm motility[58]. Gonad dysfunction is caused by a reduction in the conversion of testosterone (T) to 5-alpha-dihydrotestosterone (DHT) resulting from the low levels of 5-alpha-reductase in CD subjects, which disrupts the hypothalamus-pituitary-gonad axis[59]. Hypogonadic males who also have CD are thus more likely to develop osteoporosis, an important consideration if we bear in mind that the malabsorption associated with CD makes this disease *per se* a risk factor for osteoporosis[57]. This further highlights the significance of nutritional deficiencies in these dysfunctions and the sensitivity of the endocrine system to changes in food habits. Another aspect of hypogonadism is its association with increased levels of PRL, which in males lead not only to fertility problems, erectile dysfunction and loss of libido but also, as in females, to altered BMD[30].

Individuals with active CD have increased serum PRL levels, which correlate with the degree of atrophy of the intestinal mucosa and with the serum concentration of anti-endomysial antibodies. The presence of PRL could become a marker for the active disease if it is indeed found to have a role in modulating intestinal damage[60]. High serum levels of PRL are present not only in adults with CD but also in celiac children who do not follow the GFD[61].The above considerations suggest that in these patients the increases in PRL could also affect normal skeletal development, since in men changes in pituitary function from other causes such as, for example, tumors (prolactinoma) and drugs (antipsychotics) lead to osteopenia and osteoporosis. Bone loss in men associated

with hyperprolactinemia and hypogonadism is in fact an indirect effect of increased estrogen levels[62]. It should also be borne in mind that increased levels of PRL not associated with infertility may be indicators of a more general hypothalamus-pituitary imbalance, which may imply an increased risk of osteoporosis in men with CD[18]. The changes in PRL levels are nevertheless reversible if GFD is followed[63]. Other hormones such as GH[64], PTH[65] and Ghrelin also respond in the same way to GFD[66]. These data emphasize the importance of early diagnosis whenever there is a clinical reason to suspect the presence of CD, as for example in otherwise unexplained male osteoporosis or hypogonadism.

Celiac disease leads to a deficiency of specific micronutrients such as vitamin A, which is fundamental for the maintenance of spermatogenesis[67] and T secretion[68]. Retinols appear to act on three main types of testicular cells (Sertoli cells, germ cells and Leydig cells) of both adult and fetus[68]. A decrease in T production leads to atrophy of the accessory sex organs and, because T secretion also induces anabolic effects on these tissues[69], has negative effects on muscle and bone tissue. In addition, low circulating levels of GH lead to an altered body composition, with increased fat and reduced lean body and skeletal muscle mass[70].

Testosterone therapy for the prevention and treatment of osteoporosis in men is still a matter of controversy, but it is probable that osteoporotic men with evident hypogonadism[56], as well as those with CD with which it is often associated, can benefit from this treatment.

Strict adherence to GFD is thus necessary to avoid damage to both the reproductive system and bones. The negative effects on bones are more evident in older men with late-onset CD, for whom the risk of osteoporosis with its attendant fracture risk is greater, particularly in subjects who do not exercise regularly[71] and for whom vitamin D and calcium supplements should be recommended.

The use of pharmacological therapy (for exemple bisphosphonates) for the prevention and treatment of osteoporosis in postmenopausal women has long been recognized. The use of these agents has been traditionally based on date obtained predominantly from postmenopausal women and cases glucocorticoid-induced osteoporosis, but data are becoming increasingly available to justify their use in osteoporotic men[72]. Moreover, there is evidence that treatment with risedronate increases BMD and reduces hip fractures in elderly men[73].

CONCLUSION

Variations in the alimentary and endocrine systems in both women and men have a fundamental role in the development of osteoporosis: if these variations are combined with an inappropriate lifestyle (e.g. inadequate physical exercise, alcohol abuse or smoking), pathologies such as hyperparathyroidism, thyrotoxicosis and/or the use of drugs such as antipsychotics or corticosteroids[41], the risk of osteoporosis is increased[66]. All of these factors are able to interact with the systemic signs of CD[41]. Osteoporosis and CD are often found together and are both modulated by genetic and environmental factors, confirming their multifactorial etiopathogenesis[18,19]. The incidence of osteoporosis is higher in celiac subjects (3.4%) than in the population as a whole (0.2%) and this disease is an important extra-intestinal sign of CD. Bearing in mind that approximately 1% of the total population has CD[21], the screening of patients with idiopathic osteoporosis for CD is advisable[14]. In subjects who are also affected by endocrine-reproductive disorders, themselves a major risk factor for osteoporosis[2], this screening acquires even more importance.

Increased risk of osteoporosis should not be considered as an isolated factor, but as part of a more generalised endocrine-environmental imbalance with possible serious effects on health on account of its influence on a fairly broad range of tissues and functions and of which metabolism and bone structure are a major target. This risk of osteoporosis is further increased if the imbalance is accompanied by CD, which also causes problems for both male and female reproductive systems on account of deficiencies of important micronutrients.

One important strategy to conserve bone integrity could be the adoption of a suitable lifestyle from adolescence. Early diagnosis of CD is also indispensable so that measures to prevent and treat it, such as GFD, can be initiated in early, thereby averting an increased risk of pathologies such as osteoporosis.

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REVIEW

Human intestinal capillariasis in Thailand

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Abstract

Intestinal capillariasis caused by *Capillaria philippinensis* appeared first in the Philippines and subsequently in Thailand, Japan, Iran, Egypt and Taiwan; major outbreaks have occurred in the Philippines and Thailand. This article reviews the epidemiology, history and sources of *C. philippinensis* infection in Thailand. The annual epidemiological surveillance reports indicated that 82 accumulated cases of intestinal capillariasis were found in Thailand from 1994-2006. That made Thailand a Capillaria-prevalent area. Sisaket, in northeast Thailand, was the first province which has reported intestinal capillariasis. Moreover, Buri Ram presented a high prevalence of intestinal capillariasis, totaling 24 cases from 1994-2006. About half of all cases have consumed raw or undercooked fish. However, even if the numbers of the intestinal capillariasis cases in Thailand is reduced, *C. philippinensis* infection cases are still reported. The improvement of personal hygiene, specifically avoiding consumption of undercooked fish and promoting a health education campaign are required. These strategies may minimize or eliminate *C. philippinensis* infection in Thailand.

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Key words: Intestinal capillariasis; *Capillaria philippinensis*; Epidemiology; Prevalence; Thailand

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INTRODUCTION

Capillaria species, being round worm, are members of the superfamily Trichinelloidae. Only three major genera of this group cause human diseases: *Trichuris*, *Trichinella* and *Capillaria*. These worms have an esophagus which is surrounded by glandular cells or stichocytes. This esophageal pattern is called stichosomal esophagus. *Capillaria* species are parasites in many vertebrate animals but only three species infect humans; *Capillaria hepatica*, *C. aerophila* and *C. philippinensis*[1]. *C. philippinensis* which causes intestinal capillariasis is the most important and found to infect the human more than other species.

In 1964, the first case of *C. philippinensis* infection was found in the autopsy of a 29-year-old man from Ilocos Norte province, Northern Luzon, the Philippines, who died from intractable diarrhea[2]. After that, between 1965 and 1967, this disease spread to Pudoc West village, which is approximately 150 kilometers south of the first case. More than 1000 capillariasis cases were found and 77 died of this disease. Lately, Cross reported of the total number of intestinal capillariasis cases in Northern Luzon from 1967 to 1990 to be 1884 cases and 110 deaths[3]. In addition, sporadic cases had been reported from Japan[4], Korea[5], Taiwan, China[6-8], Indonesia[9], Iran[10] and Egypt[11-14]. *C. philippinensis* causes intermittent or continuous diarrhea leading to weight loss, abdominal pain, borborygmi, muscle wasting, weakness and edema. If the intestinal capillariasis patients are not treated, they will have severe muscle wasting, cachexia, edema and death. Most patients died from electrolyte loss resulting in heart failure and/or septicemia[3].

The earliest case of *C. philippinensis* in Thailand was reported in 1973 from an 18-mo old Thai girl[15]. Since then sporadic cases had been recorded in other parts of Thailand. In 1981, the first epidemic of intestinal capillariasis was found in Srisaket province in Northeastern part of Thailand, there had been 20 cases and 9 deaths from the disease[16]. However, even when the numbers of the intestinal capillariasis cases in Thailand are reduced, the *C. philippinensis* infection cases are still reported. Moreover, it probably contributes significantly to morbidity in humans. Therefore, the epidemiology, history and sources of *C. philippinensis* infection in Thailand, have been reviewed in this paper.

HISTORICAL REVIEW OF INTESTINAL CAPILLARIASIS IN THAILAND

The first record of intestinal capillariasis in Thailand

was in 1973. The patient was an 18-mo old Thai girl from Bangpree District of Samut Prakan province. She had diarrhea 2 or 3 times per day, for at least 6 months and edema was found for 3 d. Laboratory examination showed low albumin and potassium in blood. The feces examination revealed eggs, larvae and adults of *C. philippinensis* in her stool[15]. In 1974, Saraburi Provincial hospital reported the second case of *C. philippinensis* infection. The 46-year old man who had watery diarrhea 4-5 times a day was admitted to hospital. The result of his blood chemistry and stool examination were similar to the first case. His feces showed a large number of eggs and adults of *C. philippinensis*[17]. After the two first cases, intestinal capillariasis cases were found in other provinces in Thailand; Nakorn Panom, Surin[18], Phetchabun[19] and Maha Sarakham[20]. All patients presented edema, weight loss and had a history of watery diarrhea 3 to 6 times a day. The low level of albumin and potassium in blood were found and their feces had all stages of *Capillaria* worm. Most of them lived in raw fish eating regions and some of the cases consumed raw fresh water fish such as Koi Pla, chopped raw fish mixed with lemon juice, finely cut red onion and chili.

The first outbreak of intestinal capillariasis in Thailand was in 1981 from a small village, Phrai Bueng district, Sisaket province. This outbreak had 20 patients and 9 deaths. Most of the cases were adults who were over 20 years of age and 80% of them habited in the same household. The authors believed that man to man transmission may be a possibility because each of the cases was sequent diarrhea[16]. In 1983, Kunaratanapruk *et al* reported that 100 cases of *C. philippinensis* infection in hospital had occurred from 1979 to 1981. There were 15 cases that died from intestinal capillariasis. Seventy three percents of the patients were aged in a range between 20 and 49 years and males were more commonly infected than females, about 2.3 times[21].

After the first epidemic in 1981, a number of cases in other parts of Thailand were reported. For example, three intestinal capillariasis patients who lived in the Northeastern part of Thailand were diagnosed by Bamrasnaradura hospital[22]. Seventeen chronic diarrhea patients with *C. philippinensis* were diagnosed at Srinagarind hospital, Khon Kaen University, from 1983 to 1991. Hypoalbuminemia and ova of *C. philippinensis* in feces were found in all those cases[23]. Two intestinal capillariasis patients from the North; Phayao and Chaing Mai who had chronic watery diarrhea were admitted to the Maharaj Nakorn Chaing Mai hospital. Both patients showed eggs of *C. philippinensis* in feces and they were very fond of raw fish food[24]. The latest report, a 27-year-old man who had a history of chronic watery diarrhea for two years was found to contain *C. philippinensis* ova and worms in his jejunum[25].

LIFE CYCLE

The life cycle of *C. philippinensis* was proposed by Cross in 1992[3]. From his experimental study, larvae of *C. philippinensis* from the digestive tract of a freshwater fish (*Hypselotris bipartita*) in the endemic area were given by stomach tube to Mongolian gerbils (*Meriones unguiculatus*). Ten to eleven days post infection; larvae developed to adult stage and female worms generated larvae in 13 to 14 d. After that, these capillaries larvae developed to adults which could release unembryonated eggs within 22-24 d. Moreover, this experiment proposed that autoinfection was found in gerbils. Because they fed only two larvae from fish to gerbils but 852 to 5353 worms were recovered[26].

The biological aspect of fish as the intermediate host of *C. philippinensis* was reported by several studies. In a study in Thailand, *Cyprinus carpio* (Pla Nai), *Puntius gonionotus* (Pla Tapien Khao) and *Rasbora boraperensis* (Pla Sew), which always are prepared to Koi Pla, were fed *C. philippinensis* eggs by self feeding or forced feeding. After 10-30 d the results showed the larvae of *C. philippinensis* were recovered from fish intestines and could develop to egg producing adult in gerbils after feeding them by those larvae[27]. Likewise, Cross *et al* reported *Elotris melanosoma* (birut), *Ambassis commersoni* (bagsang) and *Apagon* sp. (bagsit), which are fresh water fish in the Philippines, were found to be intermediate hosts in experimental infection. *C. philippinensis* larvae were recovered from the digestive tract of those fresh water fish and led to intestinal capillariasis after being fed to monkeys[28]. Moreover, two specimens of *Apagon* sp. were found naturally infected with *C. philippinensis* larvae but how *C. philippinensis* can naturally infect fish is not known. The authors suggest that defecation from humans to a water resource in the endemic area is an excellent opportunity for *C. philippinensis* eggs to contact and be ingested by naturally susceptible fish[28].

Bhaibulaya *et al* reported fish-eating birds (*Amaurornis phoenicurus* and *Ardeola bacchus*) were susceptible to *C. philippinensis* infection; adults and larvae were recovered from birds' intestinal content[29]. Similarly, Cross and Basaca-Sevilla revealed *C. philippinensis* larvae from fish and experimentally infected gerbils could develop to adult males, oviparous and larviparous females in several species of fish-eating birds from Taiwan (*Nycticorax nycticorax*, *Bubulcus ibis*, *Ixobrychus sinensis*, *Gallinula chloropus*, *A. phoenicurus* and *Rostratula benghalensis*) and eggs from those birds hatched and developed to larvae and adults in fish intestines[30]. Therefore, fish-eating birds may be a natural reservoir host that upon defecation, *C. philippinensis* eggs are released to a water resource in the endemic area or eat *C. philippinensis* infected fish. These mechanisms can maintain a parasite life cycle in nature.

SYMPTOMS AND PATHOLOGY

The intestinal capillariasis patients usually present watery diarrhea weight loss, abdominal pain, borborygmi, muscle wasting, weakness, edema and laboratory examination showed low levels of potassium and albumin in blood[15,18,31] and malabsorption of fats and sugar[3,23]. Those patterns may result from *C. philippinensis* secretion of a proteolytic substance or direct penetration of the intestinal wall that causes cellular injury and dysfunction[23]. Several studies showed intestinal pathological findings in *C. philippinensis* infection which showed atrophied crypts, flattened villi, and leukocyte cell infiltration that were signs of intestinal cell injury[25,31,32]. Therefore, the destruction

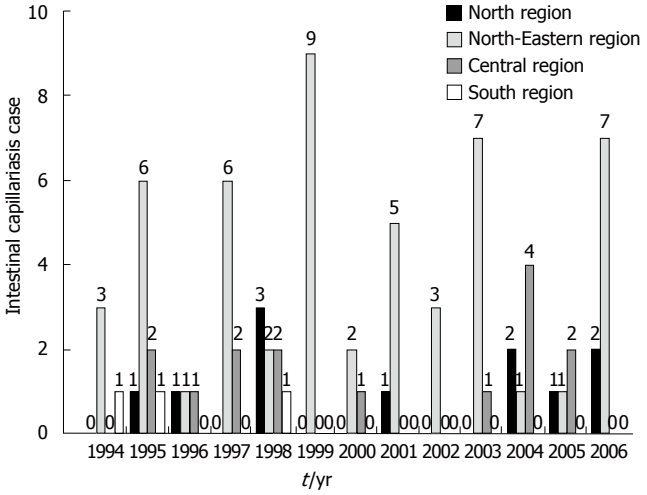


Figure 1 Intestinal capillariasis cases in Thailand from 1994 to 2006.

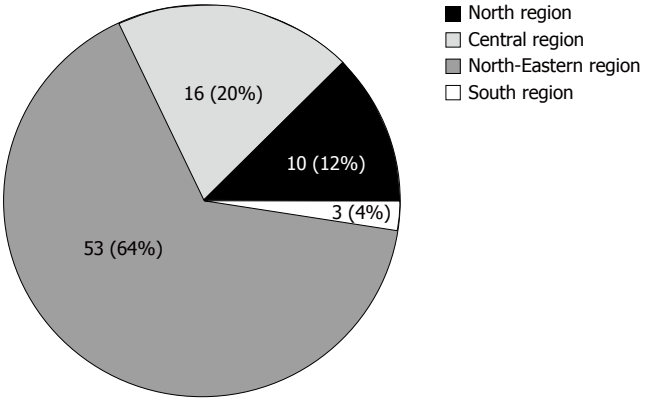


Figure 2 Intestinal capillariasis cases (82) reported in Thailand by region from 1994 to 2006.

of the intestinal cell membrane may interrupt nutrient absorption that causes weight loss in intestinal capillariasis patients. Moreover, the intestinal cells' destruction may lead to fluids, proteins and electrolytes loss because those intestinal cells are dysfunctional and cannot control fluids and electrolytes balance in the body[33] that results in a low level of potassium and albumin in the blood of *C. philippinensis* infection patients. The edema in patient, due to hypoalbuminemia[31] according to albumin levels, is plasma protein which controls the fluid in blood vessels by maintaining the osmotic pressure. If the osmotic pressure decreases, the plasma fluid in vessels leaks out of the capillaries into the interstitium and leads to edema in intestinal capillariasis patients[34].

DISTRIBUTION AND PREVALENCE OF INTESTINAL CAPILLARIASIS IN THAILAND

The epidemic of *C. philippinensis* infection was first recognized from Ah Huad village in a province with an incidence of 20 patients and 9 dead cases[16]. Thereafter, Kunaratanapruk *et al* recorded intestinal capillariasis cases at hospital. The reports showed 72 cases and 12 died

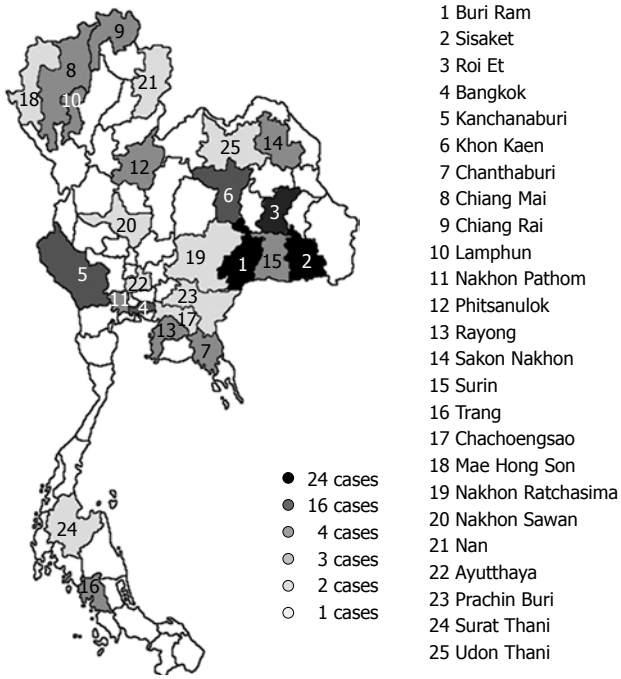


Figure 3 Twenty-five provinces in Thailand with intestinal capillariasis cases reported from 1994-2006. Each number in the map represents each province.

in 1981 and 24 cases and 2 died in 1982[21]. The annual epidemiological surveillance reports indicated that 82 accumulated cases of intestinal capillariasis were found in Thailand from 1994-2006 (Figure 1). Most of the cases occurred in 1995 and only 1 case died from intestinal capillariasis in 1996 (data not show).

Human intestinal capillariasis has occurred in many parts of Thailand, but is more common in the northeastern area. Owing to the life style, most people in this area like to eat raw fish which is believed to be the source of *C. philippinensis* infection in the area. Interestingly, no intestinal capillariasis cases were reported from the southern region during 1999 to 2006 (Figure 1). From 1994 to 2006, most of the intestinal capillariasis accumulated cases were found in the northeastern region (53 cases) and the second highest was the central region (16 cases) (Figure 2). This report was similar to previous studies which found most of the intestinal capillariasis cases in the northeastern region[16,21,23]. The provinces in the northeastern region which were found with *C. philippinensis* infection in 1994 to 2006 were Buri Ram, Khon Kaen, Nakhon Ratchasima, Roi Et, Sakon Nakhon, Surin and Udon Thani (Figure 3). Buri Ram was had the highest reported intestinal capillariasis cases which had 24 cases from 1994-2006. From 1995 through 2006, children ages less than 1 year to 9 years had been mostly infected by *C. philippinensis* (Figure 4). Contrarily, in the Philippines[3] and Egypt[35] most intestinal capillariasis cases were found in middle-aged or the active age group. This result might be caused by the child playing in the cooking area while their parent prepared food and the child eating small pieces of raw fish similarly reported by El-Dib and Doss[35]. However, working age groups (25-54 years) were still infected by *C. philippinensis* and had 38 reported cases from 1995-2006 (Figure 4).

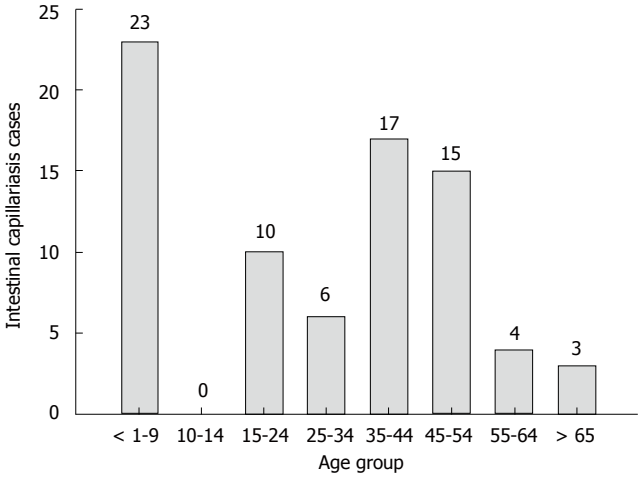


Figure 4 Intestinal capillariasis cases in Thailand by age from 1995 to 2006.

DIAGNOSIS OF INTESTINAL CAPILLARIASIS IN THAILAND

The detection of *C. philippinensis* is based on the recovery of eggs, larvae and/or adult worms in the stool of the patients. Unembryonated *C. philippinensis* eggs are peanut-shaped with flattened bipolar plugs and a striated shell. Larvae are found in the feces but difficultly identified as *C. philippinensis*[3]. An inexperienced laboratory worker may confuse with *Trichuris trichiura* eggs which are prominent mucoid bipolar plugs. The eggs of *C. philippinensis* are excreted sporadically in feces that lead to delayed diagnoses as intestinal capillariasis. Therefore, multiple stool samples may be important to early diagnosis in some cases[3]. In most of the intestinal capillariasis cases reported in Thailand eggs, larvae or adult worms in feces were found[15,16,18-20,22], but there were some patients that died from *C. philippinensis* infection in whom *Capillaria* eggs or larvae were not found in the feces[31]. However, small intestinal aspiration or biopsy may be necessary to confirm *Capillaria* infection[3]. For example, gastroduodenoscopy in a 13-year-old boy from central Thailand showed *C. philippinensis* eggs in a jejunal biopsy[32]. Moreover, jejunal mucosal biopsy and microscopic jejunal content examinations were successfully used to identify intestinal capillariasis in a 27-year-old Thai man who had negative repeated stool tests[25]. Immunodiagnosis may be a supplementary diagnostic tool which helps to detect *C. philippinensis* infection. Recently, Intapan *et al* investigated the intestinal capillaries using *Trichinella spiralis* antigen[36]. The study revealed sera from intestinal capillariasis patients were positive with *T. spiralis* antigen. This may be useful for screening of persons who have intestinal capillariasis-like symptoms before discovering *Capillaria* eggs or larvae in their stool[36].

SOURCE OF INTESTINAL CAPILLARIASIS IN THAILAND

C. philippinensis infection is mostly found in raw fish eating areas. For example, the people in Ilocos Norte province, in the Philippines, like to eat raw bagsit (*Hypseleotris bipartita*), *Capillaria* larvae containing fish, in a single bite leads to infection by *C. philippinensis*[3]. Similarly in Thailand, Koi pla and Taab Kaab are common foods that cause infection with intestinal capillariasis. Koi pla is made from chopped raw fish mixed with lemon juice, finely cut red onion and chili. Taab Kaab is made from pounded raw whole fish mixed with lemon juice, finely cut red onion and chili. These foods are favorite dishes in agricultural areas because they are easy to prepare and fresh water fish are found plentiful chiefly in these regions. In experimental study, fresh water fish in Thailand such as *C. carpio* (Pla Nai), *P. gonionotus* (Pla Tapien Khao) and *R. boraperensis* (Pla Sew), which are used to prepare Koi pla and Taab Kaab, could serve as experimental intermediate hosts for *C. philippinensis*[27]. Moreover, some people usually chew the fish freshly caught because those fish are too small to eviscerate. So the raw fresh water fish eating behavior is a major factor associated with intestinal capillariasis in Thailand.

STRATEGY FOR INTESTINAL CAPILLARIASIS CONTROL

Intestinal capillariasis is a severe disease that may lead to death unless patients are treated. The symptoms of *C. philippinensis* infection are continuous or intermittent watery diarrhea associated with severe weight loss, severe protein-loss and severe electrolyte depletion. These lead patients to die from intestinal capillariasis, so the strategy to fight the disease consists of not eating raw fish, promoting a health education program and early diagnosis are necessary to minimize or eradicate *C. philippinensis* infection in Thailand.

The health education campaign aims to promote the consumption of cooked fish and to avoid defecation into a water resource in order to eliminate *C. philippinensis* infection. The early diagnosis is necessary for treating intestinal capillariasis patients, especially improving the experience of laboratory workers and multiple stool examination must be required.

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Chaga mushroom (*Inonotus obliquus*) induces G_0/G_1 arrest and apoptosis in human hepatoma HepG2 cells

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Abstract

AIM: To investigate the anti-proliferative and apoptotic effects of Chaga mushroom (*Inonotus obliquus*) water extract on human hepatoma cell lines, HepG2 and Hep3B cells.

METHODS: The cytotoxicity of Chaga extract was screened by 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. Morphological observation, flow cytometry analysis, Western blot were employed to elucidate the cytotoxic mechanism of Chaga extract.

RESULTS: HepG2 cells were more sensitive to Chaga extract than Hep3B cells, as demonstrated by markedly reduced cell viability. Chaga extract inhibited the cell growth in a dose-dependent manner, which was accompanied with G_0/G_1-phase arrest and apoptotic cell death. In addition, G_0/G_1 arrest in the cell cycle was closely associated with down-regulation of p53, pRb, p27, cyclins D1, D2, E, cyclin-dependent kinase (Cdk) 2, Cdk4, and Cdk6 expression.

CONCLUSION: Chaga mushroom may provide a new therapeutic option, as a potential anticancer agent, in the treatment of hepatoma.

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Key words: *Inonotus obliquus*; Cell cycle; G_0/G_1 arrest; Apoptosis

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INTRODUCTION

Chaga mushroom (*Inonotus obliquus*), a white rot fungus, belongs to the hymenochaetaceae family of Basidomycetes. Chaga mushroom grows on birch trees in colder northern climates[1]. Since the sixteenth century, Chaga has been used as a folk medicine in Russia and western Siberia[2]. Recently, many polyphenolic compounds including triterpenoids, steroids, and ergosterol peroxides from *Inonotus sclerotia* have shown various biological activities, including anti-bacterial[3], hepato-protective[4], and anti-tumor[5-7] effects. However, its molecular mechanisms to the anti-tumor effects have not been well documented, in spite of its increasing usage.

Natural products including thousands of compounds that exist in fruits, vegetables, mushrooms and medical plants have been used as a source of therapeutic agents for several types of cancer or health food supplements in humans[8,9]. Especially, medicinal mushrooms, including genera *Auricularia, Flammulina, Pleurotus,* and *Trametes*, have been proven to express promising anti-tumor, immune modulating, antiviral, and antiparasitic effects[4]. It has been reported that medicinal mushrooms showed the cytotoxic effects in cancer cells by induction of apoptosis accompanying modulated cell cycle progression[10].

Cell proliferation and death are involved in the maintenance of homeostasis in normal cells; however, homeostasis is often disrupted in tumor cells with uncontrolled proliferation. Anti-tumor effects could be attributed to altered biochemical mechanisms, including inhibitions of proliferation, induction of cell cycle arrest at various cell cycle checkpoints, enhanced apoptosis, and regulation of signal transduction pathways, which are

related to altered expressions of key enzymes[11]. Cyclin-dependent kinases (Cdks), CDK inhibitors (CDKIs), and cyclins are important regulators of cell cycle progression. Also, each phase of the cell cycle is governed by different Cdks associated with their individual regulatory subunits, cyclins. The G_0/G_1 phases of the cell cycle were controlled by Cdk4/Cdk6 associated with cyclin D, late G_1 into early S phase by Cdk2 with cyclin E, S phase by Cdk2 with cyclin A, and G_2/M phase by Cdc2 kinase (CDK1) with cyclin A/cyclin B[12]. An increased expression of Cdks and cyclins accompanied by an enhanced CDK activity have been observed in most cancer cells, which could be involved in uncontrolled and unchecked cell proliferation[13]. Recently, many studies have shown that the inhibition of CDK activity, which led to cell cycle arrest, has turned out to be the most promising strategy for the discovery and design of novel anticancer agents. Therefore, cell cycle arrest and the induction of apoptosis in cancer cells become the major indicators of anti-cancer effects[14].

In this study, we investigated the anti-tumor effects of Chaga extract and the molecular mechanisms by which Chaga extract exerts inhibitive effects on liver cancer cells, HepG2 cells.

MATERIALS AND METHODS

Preparation of Chaga Mushroom Water extract

The water extract of Chaga mushroom was prepared as follows. The Chaga mushroom, *Inonotus obliquus*, collected in Russia was used in this study. Chaga mushroom was extracted under optimal water extraction condition. The crushed dry mushroom (1.00 kg) was boiled for 4 h at 100℃, cooled to room temperature, and then filtered. The extract was evaporated to dryness with a rotary vacuum evaporator and freeze-dried to give a powder (250 g). The stock solution of Chaga extract was prepared by dissolving Chaga powder in distilled water (50 mg/mL) and the experimental concentrations were diluted in the basal medium.

Cell culture and reagents

Human hepatoma cell lines, HepG2 and Hep3B, and the Chang normal liver cell line were obtained from American Type Culture Collection (Rockville, MD) and maintained in MEM (Gibco, Grand Island, NY) supplemented with heat-inactivated fetal bovine serum (FBS, Gibco), penicillin (100 U/mL) and streptomycin (100 μg/mL) under 5% CO_2 in a humidified incubator at 37℃. Propidium iodide (PI), Triton X-100, and ribonuclease-A were purchased from Sigma (St. Louis, MO, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell viability analysis

Cell viability was quantified using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). Briefly, the cells were plated in 48-well culture plates at a density of 5×10^4 cells/well and allowed to adhere at 37℃ for 12 h. The following day, various doses of Chaga extract were added to the cells and further incubated for 48 h. Then, MTT (0.5 mg) was added to 1 mL of cell suspension for 4 h. The ability of cells to form formazan crystals by active mitochondrial respiration was determined by using a Microplate reader (Titertek Multiskan, Flow Laboratories, North Ryde, Austria) after dissolving the crystals in DMSO. An empty well was used as a blank. Cytotoxicity was expressed as a relative percentage of the absorbencies measured in the control and Chaga-treated cells. Morphological changes after exposure to Chaga extract were observed by a phase-contrast inverse microscope (Olympus, Japan).

DNA cell cycle analysis

For the measurement of cellular DNA content, flow cytometric analysis was used. Briefly, 5×10^5 cells were seeded in six-well plates, and allowed to attach overnight. Cells were treated with different concentrations of Chaga extract (250, 500, 750, 1000 μg/mL) for 48 h. The cells were then harvested by trypsin treatment, washed with cold PBS, and then stained with propidium iodide (PI) solution (50 μg/mL of PI, 100 μg/mL RNase, and 0.1% Triton X-100 in PBS). The stained cells were analyzed for DNA histograms and cell cycle phase distribution by flow cytometry (FACSCalibur, BD Bioscience). Data from 10 000 cells per sample were collected and analyzed with CellQuest™ software (Becton Dickinson). To determine G_1, S, and G_2/M-phase cell populations, the settings for 2 mol/L and 4 mol/L DNA content peaks were obtained within each experiment using the G_0/G_1 arrested cell (2 mol/L) in control as the reference and applied to all samples within a given experiment.

Western blotting

Cytosolic protein extracts were prepared as in previously described methods[15]. Briefly, cells were collected by centrifugation at 300 × *g* for 5 min at 4℃ and washed with cold PBS. The cell pellet was then resuspended in 500 μL of lysis buffer (20 mmol/L HEPES- KOH, pH 7.5, 250 mmol/L sucrose, 70 mmol/L mannitol, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 10 μg/mL leupeptin, and 10 μmol/L digitonin). After 10 min incubation at 25℃, the sample was spun at 14 000 × *g* for 15 min, and the supernatant containing cytosolic proteins was stored at -70℃ until analyzed by polyacrylamide gel electrophoresis (SDS-PAGE). The protein extract was subjected to standard SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore), and probed with appropriate antibodies as described individually in the figure legends. The bound primary antibody was detected by using appropriate horseradish peroxidase-conjugated secondary antibody, and the reaction band was visualized using an enhanced chemiluminescence detection kit. Western blot analysis was done using primary antibodies against pRb, p53, p27, Cyclin E, D2, D1, Cdk2, 4, 6, pro-caspase 3, and β-actin (Santa Cruz Biotechnology) at optimal dilution. β-actin was used as an internal control to confirm that the amounts of protein load were equal.

Statistical analysis

The data shown are a summary of the results from at least three experiments and are presented as the mean ± SD. Statistical evaluation of the results was performed by one-

way ANOVA. The results were considered significant at a value of $P < 0.05$, $P < 0.01$.

RESULTS

Cytotoxicity of Chaga extract on HepG2 cells

To evaluate the effects of Chaga extract on cell proliferation and morphology of hepatoma cells, we first determined the antiproliferative effects of Chaga extract on human hepatoma cells, HepG2 and Hep3B, as well as immortalized normal liver cells, Chang. The cells were exposed to various doses of Chaga extract for 48 h and cell viability was determined by MTT assay. As shown in Figure 1A, the survival curve showed that Chaga extract had dose-dependent cytotoxic effects on HepG2 and Hep3B cells; however HepG2 cells were more sensitive to Chaga extract than Hep3B cells (Figure 1A). Within 48 h after exposure to 750 μg/mL of Chaga extract to HepG2, cell viability was reduced by 60% of control ($P < 0.01$). After treatment with 1000 μg/mL of Chaga extract for 48 h, cell viabilities of HepG2 and Hep3B were 36% and 67%, respectively. In contrast, Chang normal liver cells were not markedly affected by the treatment with Chaga extract (1000 μg/mL) for 48 h (Figure 1A). Taken together, these data indicated that Chaga extract significantly exerted apoptotic death in hepatoma cells, such as HepG2 and Hep3B cells. We next examined whether the cytotoxicity of Chaga extract is related to the induction of apoptosis. HepG2 cells were treated with 750 μg/mL of Chaga extract for 48 h, and morphological change of the cells was assessed using light microscopy. Chaga extract-treated cells (Figure 1B) showed a distinct decreased cell population compared with control cells.

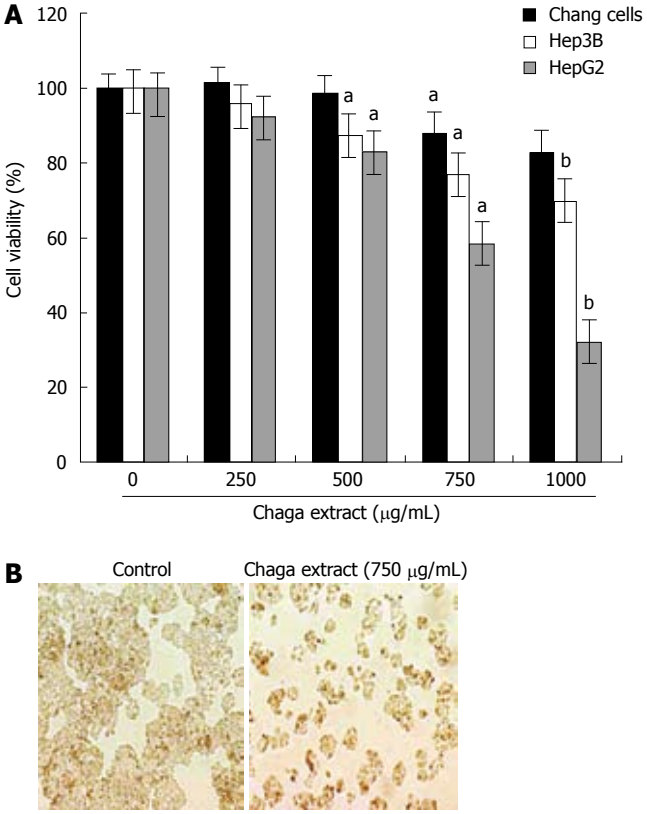


Figure 1 Cytotoxic effect of Chaga extract on human hepatoma cells. **A**: The various cells, HepG2, Hep3B, and Chang normal liver cells, were treated with various doses of Chaga extract for 48 h. Cell viability was measured by MTT assay. Data represented as means ± SD of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs* control cells; **B**: The morphological changes of HepG2 cells after exposure to Chaga extract (750 μg/mL) for 48 h. The photomicrography was taken under a phase contrast microscope (× 200).

Apoptotic cell death of HepG2 by Chaga extract

Since the cell growth and inhibition are both tightly regulated by cell cycle control[16], next, we investigated the possible inhibitory effect of Chaga extract on cell cycle distribution by flow cytometry analysis. Representative histograms for cell cycle distribution in HepG2 cells following exposure to various concentrations of Chaga extract are shown in Figure 2A. The effects of Chaga extract on HepG2 cell cycle distribution are summarized in Figure 2B. As shown in Figure 2B, treatment of HepG2 cells with 750 μg/mL of Chaga extract for 48 h resulted in a higher number of cells in the G_0/G_1 phase (75.3%) compared to media control (62.1%). This increase was coupled with the decreased percentage of cells in S phase. After a 48 h treatment, the percentage of S phase in Chaga extract-treated cells was 2.78%, whereas 9.25% in control cells. In addition, flow cytometry analysis also revealed the effect of Chaga extract on the induction of apoptosis: increase of cell percentage in the sub-G_0/G_1 phase. As shown in Figure 2C, the percentage of the sub-G_0/G_1 fraction in Chaga extract-treated cells was increased in a dose-dependent manner, indicative of apoptotic cell death. In a while, caspases are activated during apoptosis by proteolytic processing at specific aspartate cleavage sites[17]. Therefore, we determined whether the induction of apoptosis by Chaga extract resulted in an activation of caspase-3, which is a key caspase activated in apoptotic cell death (Figure 2D). As shown in Figure 3D pro- caspase-3 was markedly decreased in Chaga extract-treated HepG2 cells. These data suggest that inhibition of cell proliferation or induction of cell death in HepG2 cells by Chaga extract may be exerted by the induction of G_0/G_1 phase arrest and subsequent apoptotic process accompanying caspase-3 activation.

Effect of Chaga extract on the modulators of cell cycle

Tumor suppressor gene p53 has been shown to play an important role in the induction of cell cycle arrest and apoptosis[18]. Also, pRB has been known to play a critical role in the G_1 to S phase cell cycle transition[19]. Since the inhibitory mechanism of Chaga extract on cell proliferation might be caused by the expression of negative regulators of the cell cycle, we evaluated the protein expression of p53, pRb, and p27. As shown in Figure 4, we found that p53 and pRb protein was obviously decreased by Chaga extract in a dose-dependent manner in HepG2 cells (Figure 3). The p27, a specific inhibitor of CDKs, is mainly involved in G_1/S transition of the cell cycle. The p27 was also markedly down-regulated by treatment with Chaga extract in a dose dependent manner (Figure 3).

Down-regulation of G_0/G_1 regulatory proteins

To analyze the underlying biochemical mechanisms

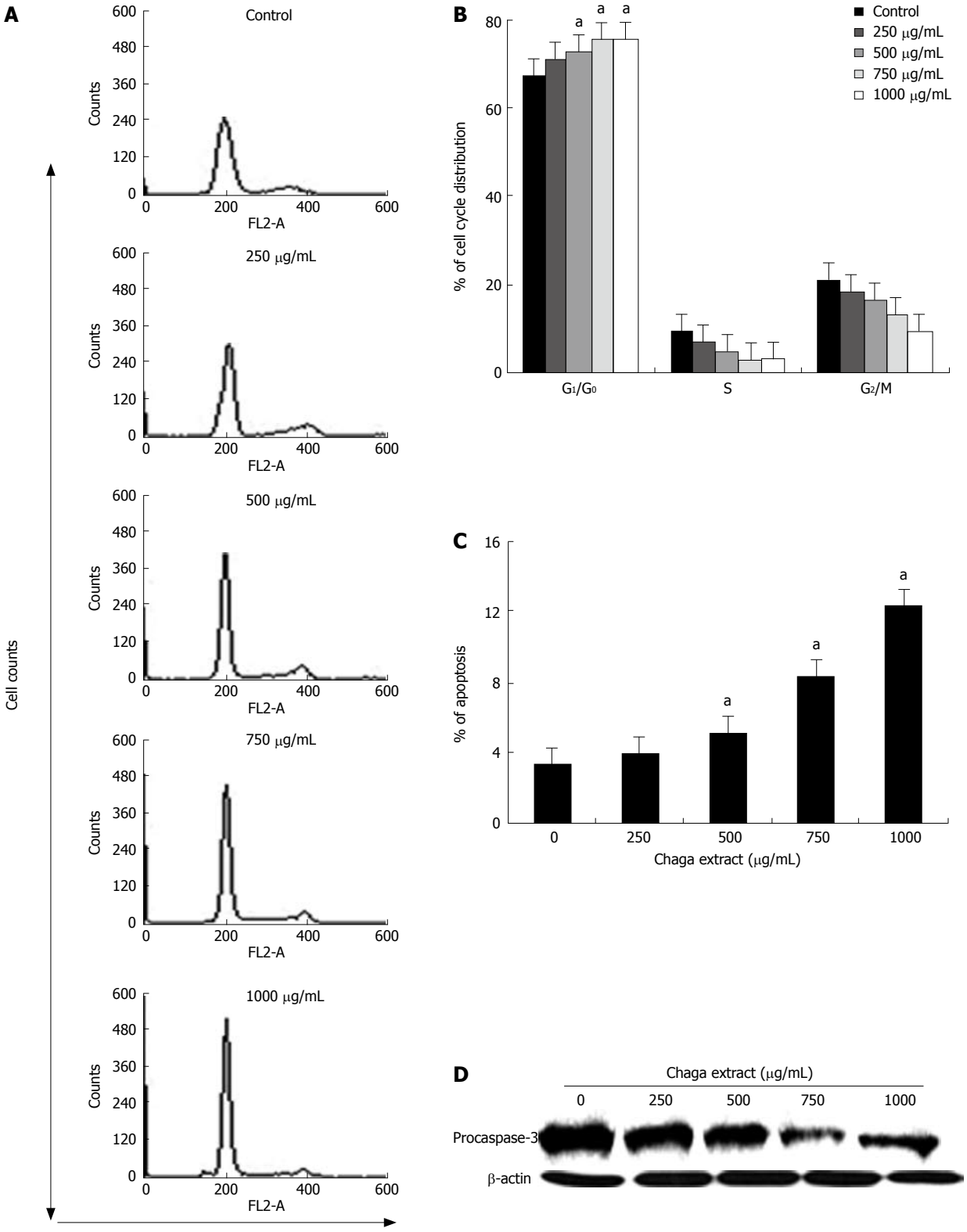


Figure 2 Apoptotic cell death of HepG2 by Chaga extract. The effect of Chaga extract on cell cycle progression of HepG2 cells was determined. HepG2 cells were treated with various doses of Chaga extract for 48 h. Then, cells were harvested and washed with cold PBS. **A**: Cellular DNA content was stained with PI solution and flow cytometric analysis was performed to analyze the cell cycle distribution. Cell cycle histogram was shown; **B**: A summary of cell cycle distribution data in histograms. Data represented as means ± SD of three independent experiments. $^{a}P < 0.05$, *vs* control cells; **C**: The percentage of subG$_0$/G$_1$ phase was represented as % of apoptosis. Data represented as means ± SD of three independent experiments. $^{a}P < 0.05$ *vs* control cells; **D**: Caspase-3 activation, which was measured by cleavage of procaspase-3, in Chaga extract-treated HepG2 cells was determined by western blot analysis.

involved in the regulation of the G$_0$/G$_1$ cell cycle arrest, we investigated the effect of Chaga extract on the protein levels of the Cdks and cyclins during G$_0$/G$_1$ cell cycle arrest progression. As shown in Figure 4A, treatment of HepG2 cells for 48 h with Chaga extract resulted in a prominent decrease in the expressions of Cdk2, Cdk4, and Cdk6 in a dose-dependent manner. Of note, a marked reduction in Cdk2 and Cdk6 was observed even at a dose

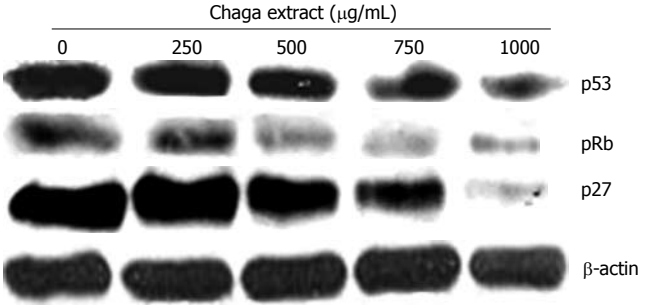


Figure 3 Effect of Chaga extract on the modulators of cell cycle such as p53, pRb, and p27. Western blot analysis for p53, pRb and p27 was performed using specific antibodies with β-actin as a loading control.

of 250 μg/mL of Chaga extract. In addition, Chaga extract markedly decreased the expression of Cyclin D1, D2, and E (Figure 4B). These observations suggest that the down-regulation of the levels of cyclin/Cdk proteins in Chaga extract-treated HepG2 cells is closely related to the induction of G_0/G_1 arrest of cell cycle progression.

DISCUSSION

Several anti-cancer drugs or compounds for cancer treatment have been found in natural products. In this study, we found that aqueous extracts of Chaga mushroom (*Inonotus obliquus*) caused a growth inhibition of human hepatoma HepG2 cells, and this was closely associated with the arrest of the cell cycle in G_0/G_1 phase and induction of apoptosis.

The eukaryotic cell cycle is regulated by signal transduction pathways mediated by a series of cell-cycle regulators. Cyclins are positive regulators of cell cycle progression and function by forming a complex with CDKs. CDK inhibitors are negative regulators of the cell cycle and bind to cyclin-CDK complexes and inhibit the activity of those complexes[20]. Therefore, induction of cell cycle arrest and apoptosis by chemopreventive drugs could be an effective approach to treat uncontrolled cell proliferation and survival in tumor cells.

In recent years, mushroom extracts have been found to have anti-inflammatory, anti-tumor properties[21]. Although it has been reported that the Chaga mushroom and other mushrooms have therapeutic effects such as anti-tumor, anti-inflammatory and hepatoprotective effects[4], the mechanisms of anti-inflammatory and anti-tumor effects of the Chaga mushroom have not been clearly elucidated[22]. In the present investigation, we showed that the water extract of the Chaga mushroom, which has been used in the treatment of cancers and digestive system diseases[2], significantly inhibited the viability and proliferation, and induced apoptotic cell death in human hepatoma HepG2 cells (Figure 1A), but this effect was not found in Chang cells, the human immortalized non-tumor cell line (Figure 1C). These data indicate that Chaga extract has selective cytotoxic effects on human hepatoma cells. This selectivity may be the great advantage of the Chaga extract for therapeutic or preventative use in cancer treatment. Though there were several reports that many natural products induce cell cycle arrest in various cell

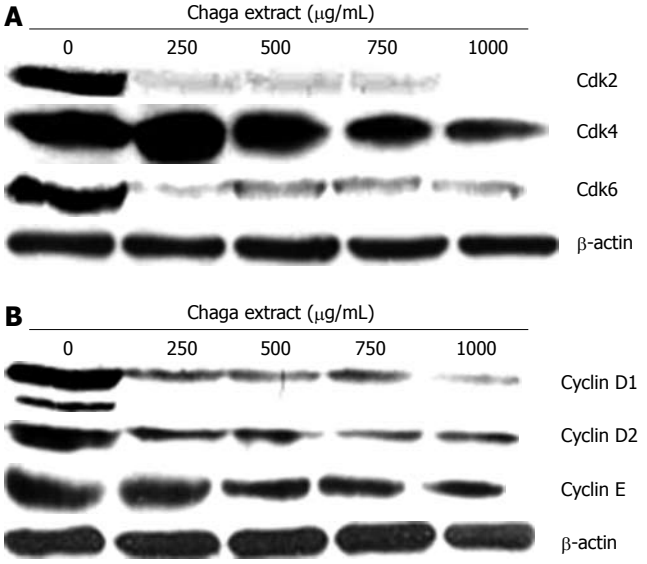


Figure 4 Effect of Chaga extract on the protein levels of G_0/G_1 regulatory Cdks and cyclin in HepG2 cells. The effect of Chaga extract on the protein levels of G_0/G_1 regulatory Cdks and cyclin in HepG2 cells was determined by western blot. Western blot analysis for Cdks (**A**: Cdk2, Cdk4, and Cdk6) and Cyclins (**B**: Cyclin D1, Cyclin D2, and Cyclin E) was performed using specific antibodies with β-actin as a loading control.

cycle phases, this study is the first report that reveals the inhibitory effect of Chaga extract on the cell cycle in cancer cell lines. In this study, flow cytometric analysis clearly revealed that HepG2 cells were dose-dependently arrested by Chaga extract at the G_0/G_1 phase of the cell cycle (Figure 2A).

The blockade of survival pathways or the induction of apoptosis pathways by anti-cancer agents prevents the proliferation of cancer cells, which may be exploited for cancer therapy[23]. p53 is a tumor suppressor gene encoding a transcription factor. Its tumor-suppressive activity involves inhibition of cell proliferation through cell cycle arrest and/or apoptosis. Mutation in p53 occurs in more than half of human cancers[24-26]. Therefore, the activation of p53 by anticancer agents may induce the cell cycle arrest and apoptosis in cancer cells leading to the inhibition of tumor progression. HepG2 has a functional p53 (wt p53), however Hep3B does not have a functional p53 (delete p53)[27]. In our study, HepG2 cells were more sensitively damaged by Chaga extract than Hep3B cells (Figure 1). Therefore, we speculated that wt p53 plays an important role on the apoptosis-inducing effect of Chaga extract in HelpG2 cells. However, our data did not show whether the activation of p53 may mediate the cytotoxicity and cell cycle arrest in Chaga extract-treated HepG2 cells, because wt p53 was down-regulated by Chaga extract (Figure 4). Interestingly, Park *et al* (2006)[28] showed that 6-gingerol, a major phenolic component in ginger, induced G_0/G_1 arrest with up-regulation of $p21^{WAF1}$ expression and down-regulation of p53/pRb expression in pancreatic cancer cells. In the present study, the down-regulation of pRb and p27 protein expression as well as p53 was detected in the Chaga extract-treated HepG2 cells. Our major question remaining to be addressed is that the down-regulation of p53, pRB, and p27 protein, are closely associated with the

terminal differentiation or the other signaling pathways levels. However, unfortunately, this question cannot be addressed at the present time.

Eukaryotic cell cycle progression involves sequential activation of Cdks, whose activation is dependent upon their association with cyclins. G_1-phase arrest of cell cycle progression provides an opportunity for cells to either undergo repair mechanisms or proceed by the apoptotic pathway[29]. It has been suggested that Cdk activities control G_1/S transition in mammalian cells[30]. Both D- and E-type cyclins are known to be important regulators in G_1/S control, even though some reports raised the possibility that they have other distinct roles[31]. In our results, we found that treatment with Chaga extract causes a significant decrease in the expression of cyclin D1, D2 and E and Cdk 2, Cdk4, Cdk6 in HepG2 cells. Thus, we speculated that the reduction of cyclin or Cdk expression resulted in the blocking of cyclin/Cdk complex formation and that lowered the levels of pRb (Figure 3). When Rb proteins remain in an unphosphorylated form, E2F cannot be activated and the cells fail to enter the S phase[28]. Based on the data (Figure 4), it seems that cyclin D1 and Cdk2, and Cdk6 are responsible for most of the cell cycle arrest observed in response to Chaga extract because these regulators are effectively deceased at the lowest dose of Chaga extract (250 μg/mL).

Taken together, we concluded that Chaga extract induced the growth inhibition, G_0/G_1-phase arrest, and apoptosis in human hepatoma HepG2 cells, but not in normal Chang liver cells. The present results can provide new hope for chemotherapy of hepatoma cancer. Furthermore, an improved understanding of the interactions between phytochemicals with the genes that are critical to the regulation of cancer cell growths will provide strong armaments to cancer therapy.

COMMENTS

Background

Chaga mushroom (*Inonotus obliquus*), a white rot fungus, belongs to the hymenochaetaceae family of Basidomycetes. Since the sixteenth century, Chaga has been used as a folk medicine in Russia and western Siberia. Chaga mushroom have shown various biological activities, including anti-bacterial, hepato-protective, and anti-tumor effects. However, its molecular mechanisms to the anti-tumor effects have not been well documented, in spite of its increasing usage. Therefore, we investigated the anti-tumor effects of Chaga extract and the molecular mechanisms by which Chaga extract exert inhibitive effects on liver cancer cells, HepG2 cells.

Research frontiers

Eukaryotic cell cycle is regulated by signal transduction pathways mediated by a series of cell-cycle regulators. Therefore, induction of cell cycle arrest and apoptosis by chemopreventive drugs could be an effective approach to treat uncontrolled cell proliferation and survival in tumor cells. Though there were several reports that many natural products induce cell cycle arrest in various cell cycle phases, this study is the first report that reveals the inhibitory effect of Chaga extract on cell cycle in cancer cell lines.

Innovations and breakthroughs

Chaga extract has selective cytotoxic effects on human hepatoma cells. Many reports indicated that cell cycle regulatory proteins such as p53 and p27, are up-regulated in G_0/G_1 cell cycle arrest. Nevertheless, we observed that Chaga extract induced the down-regulation of p53, pRb, and p27 expression in G_0/G_1 arrest. However, the precise molecular mechanisms involved in the decrease of the cell cycle regulator proteins, p53, pRb, and p27, are still unclear.

Applications

Chaga mushroom may provide a new therapeutic option, as a potential anticancer agent, in the treatment of hepatoma.

Peer review

The authors investigated the anti-proliferative and apoptotic effects of Chaga mushroom (*Inonotus obliquus*) water extract on human hepatoma cell lines, HepG2 and Hep3B cells. Chaga mushroom may provide a new therapeutic option, as a potential anticancer agent, in the treatment of hepatoma.

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LIVER CANCER

Preventive effect of regional radiotherapy with phosphorus-32 glass microspheres in hepatocellular carcinoma recurrence after hepatectomy

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Abstract

AIM: To evaluate the preventive effects of phosphorus-32 glass microspheres (P^{32}-GMS) in the recurrence of massive hepatocellular carcinomas (HCCs) after tumor resection.

METHODS: Twenty-nine patients with massive HCCs received local P^{32}-GMS implantation after liver tumors were removed, while the other 38 patients with massive HCCs were not treated with P^{32}-GMS after hepatectomies. The radioactivity of the blood, urine and liver were examined. The complications, HCC recurrence and overall survival rates in the patients were analyzed.

RESULTS: P^{32}-GMS implanted in the liver did not cause systemic absorption of P^{32}. There were no significant differences of postoperative complications between the patients with and without P^{32}-GMS treatment. The short-term (six months and 1 year) and long-term (2, 3 and over 3 years) recurrence rates in patients who received P^{32}-GMS radiotherapy were significantly decreased, and the overall survival rates in this group were significantly improved.

CONCLUSION: P^{32}-GMS implantation in the liver can significantly decrease the postoperative recurrence and improve the overall survival in HCCs patients after hepatectomy. This therapy may provide an innovative method in prevention of HCC recurrence after operation.

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Key words: Hepatocellular carcinoma; Recurrence; Phosphorus-32 glass microspheres; Hepatectomy

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in southeastern China. The mortality of this disease has ranked as the second highest in cancer nationwide and responsible for an estimated one million deaths annually since the 1990s, and its morbidity is increasing among males[1,2]. Currently, the prognosis of HCC mainly depends on the results of surgical treatment of the tumors. The surgical treatments include the tumor resection and liver transplantation, which offer the only chance for radical treatment of HCC patients[3].

Clinically, with the characteristics of rapid infiltrating growth, early local and/or distant metastasis and high postoperative recurrence, the massive HCCs are usually diagnosed when they are at advanced stages with poor prognosis. The recurrence rate of intrahepatic carcinomas varies from 36.8% to 82%, which caused death in about 50% of the patients[4-7]. Prevention of cancer recurrence and metastasis after curative surgical resection has become a key issue for improvement of the surgical outcome and long-term survival of the patients.

In the past decades, many adjuvant interventional therapies have been continuously extended as a result of the technical development of locoregional approaches for HCCs. The combined multimodal therapies have played important roles in preventing postoperative recurrence of HCCs. Recently, nuclide labeled with undegradable micro-carriers has become a new radioactive therapy for malignant liver cancers.

Phosphorus-32 (P^{32}) is a radioisotope which emits

relatively high energy beta particles as it decays. Recent experimental studies and clinical trials showed that intrahepatic arterial application of P^{32} labeled with undegradable glass microsphere could cause death of tumor cells in the animal model and reduce the size of the tumor[8-10]. However, this therapy was only reported for unresectable liver cancers. Since the postoperative recurrence of HCC is closely related with the peritumor micrometastasis and portal vein tumor invasion[11,12], development of the local radiotherapy may provide a method to prevent the recurrence of HCCs after radical hepatectomy. In this study, the outcome and complications of implantation of phosphorus-32 glass microspheres (P^{32}-GMS) in a series of patients with massive HCCs were analyzed after hepatectomy. We present for the first time the preventive effect of the local radiation therapy with P^{32}-GMS in HCC recurrence after radical hepatectomy.

MATERIALS AND METHODS

P^{32}-GMS preparation

P^{32}-GMS was generated by activation of standardized glass microspheres through a nuclear-chemical reaction, in which the nonradioactive P^{31} (P^{31}-GMS, cold sphere) was transformed into the radioactive P^{32} glass microsphere (provided by Nuclear Power Research Institute of China). The diameter of glass sphere is between 46 μm and 76 μm, with radioactivity of 550-3700 MBq/g per unit and P^{32} elution rate of less than 0.1% within 30 d. The radioactive nuclide purity was more than 99% with the physical half-life 14.28 d, and the average β ray energy was 0.695 MeV (maximum energy 1.711 MeV) per disintegration, and soft tissue penetration distance was 3.2 mm in average (maximum 8.0 mm). The P^{32}-GMS suspension was prepared by mixing P^{32}-GMS with 1 mL super-liquid iodized oil or 50% glucose solution to a concentration of 100 g/L on oscillator.

Implantation of P^{32}-GMS

The P^{32}-GMS suspension was soaked into a piece of the absorbable gelatin sponge. After hepatectomy, the gelatin sponge was buried within the liver resection surface during closure. The dose of P^{32}-GMS implantation depends on the size of the tumor and calculated by the formula: $Dt = 34.6 \times T \times C \times \sum\Delta i\ (1\text{-}E^{-0.693t/T})$. The mean dose of radiation for the patients was 5.27 ± 0.27 Gy. The total tissue absorbed dose after radioactive P^{32} disgregation was 135.18 Gy in average.

Patients

From June 1999 to September 2003, 67 patients were diagnosed as having HCCs and underwent hepatectomy. Among them, 29 patients received P^{32}-GMS radiotherapy after hepatectomy (group A), and the other 38 patients without radiotherapy after operation served as controls (group B). The patients' age, sex, preoperative intervals, tumor size, hepatitis B virus (HbsAg) infection rate, positive alpha-fetoprotein (α-FP), microvenous tumor invasion in both groups were evaluated.

After surgery, the liver radioactivity distributions were measured by a photo-emission computed tomography (ECT) on postoperative d 1, 7 and 14. The radioactivity of blood and urine were also measured daily with γ-ray counter in the first postoperative week and on postoperative d 14. The postoperative complications including death, subdiaphragm infection, bile leakage, leucopenia, jaundice, and pleura effusion and/or ascites were analyzed in each group. The survival of the patients (six months, 1 year, 2 years, 3 years and over 3 years) and cancer recurrence rates were compared between the two groups.

Table 1 General information of patients in groups A and B

	Group A (n = 29)	Group B (n = 38)	P value
Age (yr)	53.83 ± 2.20	55.26 ± 6.67	$P > 0.05$
Sex (male/female)	24/5	32/6	$P > 0.05$
Tumor size (cm)	7.58 ± 2.50	7.76 ± 3.13	$P > 0.05$
Preoperative interval (d)	23.34 ± 2.45	21.79 ± 4.19	$P > 0.05$
HbsAg positive rate (%)	75.9	81.58	$P > 0.05$
α-FP positive rate (%)	65.5	72.97	$P > 0.05$

Statistical analyses

The data were expressed as the mean ± SEM and mean ± SD. Differences between groups were compared using Kaplan-Meier method, un-paired Student's t test, and Chi-square. A P value less than 0.05 was defined as significant. All data were analyzed using SPSS11.5 statistics software (SPSS Inc., Chicago, IL).

RESULTS

Patients

The general information of the HCC patients is shown in Table 1. The average tumor size was 7.58 ± 2.50 cm in group A and 7.76 ± 3.13 cm in group B. There was no significant difference between the two groups. The positive rates of alpha-fetoprotein (α-FP) were 65.5% and 72.97% in groups A and B, respectively ($P > 0.05$). The postoperative pathologic examination revealed that the microvenous tumor invasion rate was 10.3% in group A and 13.16% in group B, respectively ($P > 0.05$). The comparisons of other parameters also showed no significant differences between the two groups.

Radioactivity

In patients of group A, the β rays of P^{32} in liver was detected by ECT (Figure 1), and the postoperative disgregation of radioactivity is shown in Figure 2. The radioactivity of P^{32} decreased on postoperative d 7 and d 14 when compared with postoperative d 1. The half life of P^{32} was about 14 d. The disgregation of the P^{32} radioactivity in liver matched the dynamic eradication of the radioisotope P^{32}.

The radioactivity in blood and urine was also measured in group A. The results showed that there was no significant radioactivity enhancement in blood and urine after P^{32}-GMS was implanted (Figure 3), which indicated that P^{32} was not systemically absorbed in the patients.

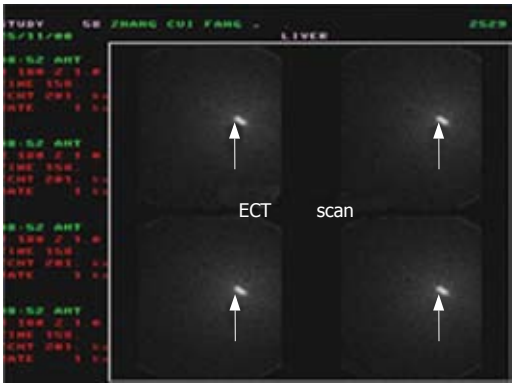


Figure 1 Liver ECT scan. Arrow: The site where P^{32}-GMS was implanted.

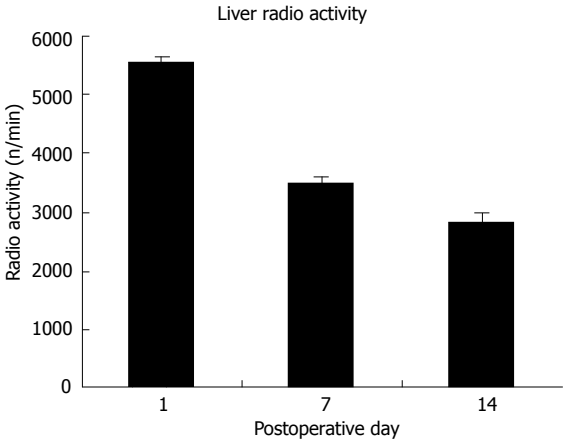


Figure 2 P^{32} Radioactivity in liver of the patients with P^{32}-GMS (n/min). The radio activity of P^{32}-GMS decreased on POD 7 and POD 14 as compared with POD 1. (POD = postoperative day).

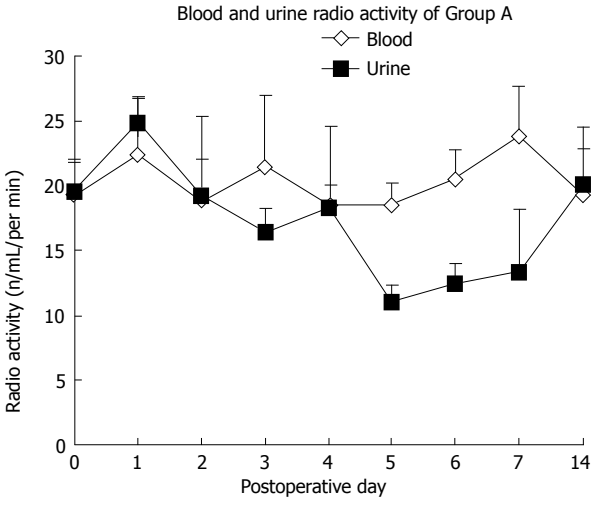


Figure 3 Radioactivity in blood and urine of the patients with P^{32}-GMS (n/mL/min). The radio activity in blood and urine were low. It was familiar with the sample without P^{32}-GMS implanted (d 0).

Complications

There was no perioperative patient death within 30 d after surgery in both groups. After hepatectomy, liver failure occurred in 3 patients (10.3%) of group A and 3 patients (7.89%) of group, respectively. The morbidity of other postoperative complications was also compared between the two groups (Table 2). There were no significant differences between the two groups.

Table 2 Postoperative complications in patients with and without P^{32}-GMS radiotherapy, *n* (%)

Complications	Group A (*n* = 29)	Group B (*n* = 38)	*P* value
Liver failure	3 (10.3)	3 (7.89)	$P > 0.05$
Perioperative death	0	0	$P > 0.05$
Subdiaphragm infection	1 (3.45)	2 (5.26)	$P > 0.05$
Bile leakage	0	0	$P > 0.05$
Leukopenia	0	0	$P > 0.05$
Jaundice	1 (3.45)	2 (5.26)	$P > 0.05$
Liver wound rupture	0	0	$P > 0.05$
Pleural effusion and/or ascites	5 (17.24)	5 (13.15)	$P > 0.05$

Table 3 HCC recurrence rates in patients with and without P^{32}-GMS, *n* (%)

Postoperative intervals	Group A (*n* = 29)	Group B (*n* = 38)	*P* value
6 mo	4 (13.8)	15 (39.5)	$P = 0.021^a$
1 yr	7 (24.1)	20 (52.6)	$P = 0.018^a$
2 yr	13 (44.8)	29 (76.3)	$P = 0.008^b$
3 yr	18 (62.1)	33 (86.8)	$P = 0.018^a$
Over 3 yr	18 (62.1)	33 (86.8)	$P = 0.018^a$

$^aP < 0.05$; $^bP < 0.01$.

Table 4 Survival rates in patients with and without P^{32}-GMS, *n* (%)

Postoperative intervals	Group A (*n* = 29)	Group B (*n* = 38)	*P* value
6 mo	28 (96.6)	35 (92.1)	$P = 0.454$
1 yr	27 (79.3)	27 (71.1)	$P = 0.024^a$
2 yr	22 (75.9)	17 (44.7)	$P = 0.010^b$
3 yr	19 (65.5)	8 (21.1)	$P = 0.000^b$
Over 3 yr	14 (48.3)	8 (21.1)	$P = 0.018^a$

$^aP < 0.05$; $^bP < 0.01$.

Recurrence rate and survival rate

The recurrent risk was estimated in both groups. The follow-up investigations showed that the short-term (6 mo and 1 year) and long-term recurrence rates at 2, 3 and over 3 years in group A were significantly reduced when compared with those who did not receive preventative radiotherapy (Table 3).

Although there was no significant difference in the six-month survival rates between two groups, the overall survival rates at 1 year, 2 years, 3 years and over 3 years in group A were significantly higher than those patients without radiotherapy (Table 4). Figure 4 shows a case of a 56-year-old woman who was diagnosed having progressive liver cancer. The patient received preventive P^{32}-GMS implantation at a dose of 6.83 Gy radioactivity after hepatectomy. There were no signs of liver radiation damage and cancer recurrence in the CT scans during the 4-year follow-up.

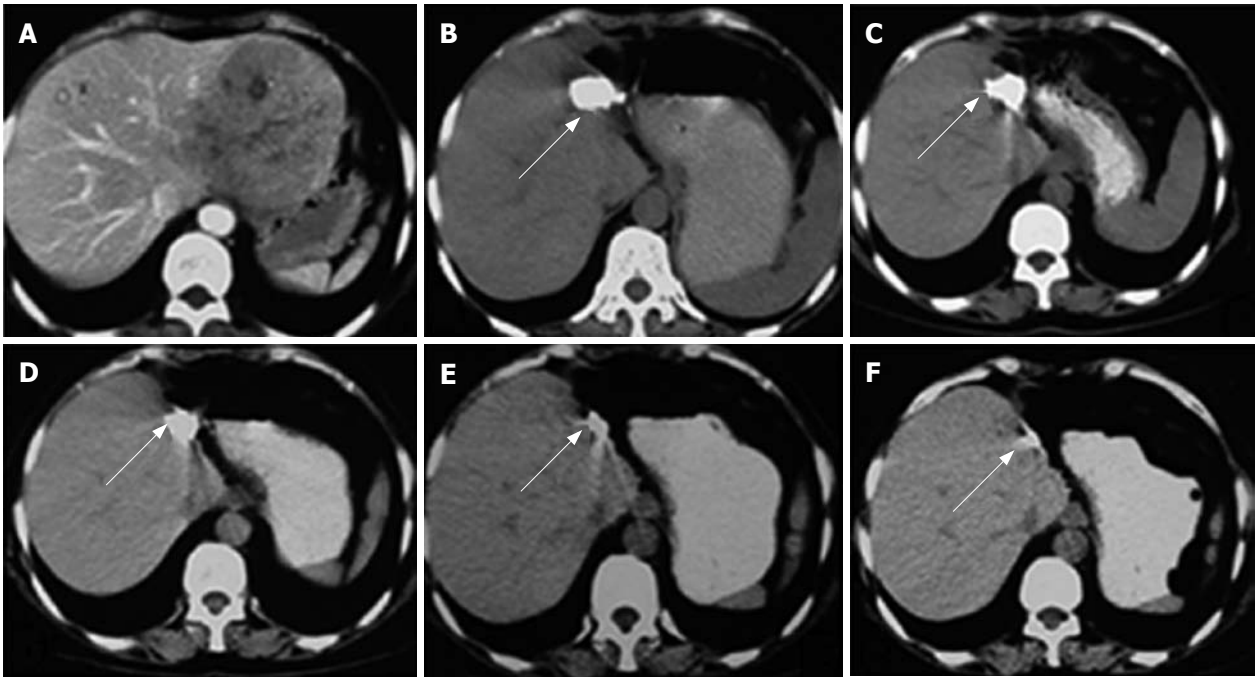


Figure 4 CT scan of the HCC patient who underwent hepatectomy and P^{32}-GMS treatments in 4 years following-up. **A**: Liver CT scan showing a progressive liver tumor prior to hepatectomy; **B**: Liver CT scan after hepatectomy; **C**: Liver CT scan at first postoperative year; **D**: Liver CT at second postoperative year; **E**: Liver CT at third postoperative year; **F**: Liver CT at fourth postoperative year. Arrows: implanted P^{32}-GMS.

DISCUSSION

The radical hepatectomy is now considered a major choice for the patients with resectable HCC. Recent development of surgical techniques and perioperative management have remarkably reduced the death that is caused by surgery[13,14]. In the past two decades, the advanced techniques of the curative tumor resection has also significantly improved the long-term survival of the patients. A recent report has shown that about a 50% survival rate has been achieved for the patients treated with surgery in a five-year follow-up[15]. However, the high postoperative recurrence rate is still a major problem for the long-term survival of the patients with HCCs after hepatectomy.

It has been reported that the 5-year recurrence rate of HCCs after curative resection ranges from 38% to 61.5%[16,17]. The recurrent peak is found at six months or 1 year after operation and most of the early postoperative recurrence is intrahepatic metastasis[18], which is attributed to the characteristics of vascular invasion propensity of HCCs. The appearance of tiny tumors through venous invasion in the liver is the main sign for potential HCC recurrence after surgery[19-29]. The intrahepatic metastasis of the tumor cells through the portal venous system is believed to be an important mechanism for HCC recurrence[24,30,31].

Since the tumors usually are located close to the major vessels or the severe liver cirrhosis coexists, extensive excision of liver frequently results in hepatic failure. The microvenous tumor embolus or existence of the residual tumor in the margin of remnant liver due to inadequate resection may cause the early postoperative recurrence of HCCs. Therefore, combination of adjuvant interventional therapies may play an important role in preventing postoperative recurrence of HCCs.

Several adjuvant interventional therapies for preventing postoperative recurrence of HCCs were investigated around the world. Some adjuvant approaches were effective in reducing the postoperative recurrence of HCC and improving survival of the patients. The postoperative transcatheter arterial chemoembolization (TACE) was shown to postpone the peak of recurrence rate in patients from 6 to 12 mo. However, the recurrence rate in the group with TACE treatment was even higher than that of the control group without TACE at a 18-mo follow-up[32]. One study showed that postoperative application of interferon alpha might postpone the recurrence of the HBV-related HCC after curative resection and improve the overall survival of patients[33]. In another study, postoperative intra-arterial injection of iodine131 labeled lipiodol (131I-Lip) was found to decrease the HCC recurrence rate in patients after hepatectomy, improving the 1-, 2-, and 3-year survival rates when compared with the survival rates in the control group receiving no 131I-Lip treatment[34]. The adjuvant chemotherapy was not effective in preventing the postoperative recurrence[35].

The mechanisms of the anticancer effect of P^{32}-GMS have been discussed. The experimental studies showed that the β-ray energy could directly destroy or injure the DNA of tumor cells while inducing death of the cells. Simultaneously, the β-ray irradiation could induce generation of several types of free radicals and superoxides, which could damage the tumor cells[36]. The β-ray was also found to induce the apoptosis of tumor cells[37,38]. In an experimental study in the nude mice model, intra-mass injection of P^{32}-GMS into the implanted human liver cancer was found to cause the death of many

tumor cells[9]. In some studies, intra-tumor injection of P^{32} chromic phosphate resulted in remarkable regression of the tumors[39,40]. The current study was to examine if local implantation of P^{32}-GMS in the liver after hepatectomy could decrease the risk of postoperative tumor recurrence.

In our study, the short-term (six months and 1 year) and the long-term (2, 3 and over 3 years) recurrence rates were significantly decreased in patients who received P^{32}-GMS radiotherapy. Decrease of tumor recurrence results in significant increase of survival of the patients. These findings demonstrated that the implantation of P^{32}-GMS in the liver after hepatectomy can provide a preventive effect in postoperative recurrence of HCC and improve the overall survival of the patients. This preventive effect of P^{32}-GMS is probably through damaging the tumor cells from the microvenous tumor embolus in the margin of remnant liver. However, the implantation of P^{32}-GMS under liver incision edge may not prevent the tumor recurrence from the distant intrahepatic metastasis prior to hepatectomy or the multi-centric HCCs.

Our study has also shown that there were no significant differences in postoperative complications between the patients receiving P^{32}-GMS radiotherapy and the patients without radiotherapy after surgery. No side effects of P^{32}-GMS implantation were observed and no radiation was detected in both blood and urine samples in the patients with P^{32}-GMS treatment. These results further confirmed the safety of local implantation of P^{32}-GMS in the liver.

In conclusion, P^{32}-GMS implantation in the liver after the resection of HCC can significantly decrease the postoperative recurrence of HCC, and improve the overall survival in the patients. This radiotherapy will not cause any side effects and complications, and may provide an innovative method for the prevention of HCC recurrence after hepatectomy.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies related to a high mortality globally. In recent years, the surgical treatments, including the tumor resection and liver transplantation, offer the only chance for radical treatment of HCC patients. Clinically, the massive HCCs are usually diagnosed at advanced stages, and the prevention of cancer recurrence and metastasis after curative hepatectomy has become a key issue for the improvement of the overall survival of the patients.

Research frontiers

In recent years, nuclide labeled with nontoxic and undegradable micro-carriers such as phosphorus-32 glass microsphere (P^{32}-GMS) has gained much attention as a new radioactive therapy for malignant liver tumors.

Innovations and breakthroughs

Some experimental studies and clinical trials have shown cytocidal effect of local intrahepatic irradiation of P^{32}-GMS in unresectable liver cancers. However, the study of evaluating the preventive effect of the local radiation therapy with P^{32}-GMS in HCC recurrence after radical hepatectomy has not been reported.

Applications

Through evaluating the pharmacology, toxicology and clinical effect, P^{32}-GMS was considered as a helpful therapeutic weapon combined with surgical and other nonsurgical treatment of liver cancer.

Terminology

P^{32}-GMS, which is transformed from nonradioactive P^{31}-GMS by activating the standardized glass microspheres through nuclear-chemical reaction, is the nuclide labeled nontoxic and undegradable micro-carriers. Recently, P^{32}-GMS and yttrium-90 glass microsphere have been successfully developed as a new radioactive medicine for treating malignant liver neoplasms.

Peer review

This report is the first study to evaluate the preventive effect of the local radiation therapy with P^{32}-GMS on HCC recurrence after radical hepatectomy. Although more studies are needed to confirm the safety and effect of the new medicine, it will be beneficial to the prognosis and treatment of HCC patients in the near future.

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COLORECTAL CANCER

Hypermethylated *SFRP2* gene in fecal DNA is a high potential biomarker for colorectal cancer noninvasive screening

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Abstract

AIM: To investigate the feasibility of detecting hypermethylated secreted frizzled-related protein 2 (*SFRP2*) gene in fecal DNA as a non-invasive screening tool for colorectal cancer (CRC).

METHODS: Fluorescence-based real-time PCR assay (MethyLight) was performed to analyze *SFRP2* gene promoter methylation status in a blinded fashion in tumor tissues and in stool samples taken from 69 CRC patients preoperatively and at the 9th postoperative day, 34 patients with adenoma $\geqslant$ 1 cm, 26 with hyperplastic polyp, and 30 endoscopically normal subjects. Simultaneously the relationship between hypermethylation of *SFRP2* gene and clinicopathological features was analyzed.

RESULTS: *SFRP2* gene was hypermethylated in 91.3% (63/69) CRC, 79.4% (27/34) and 53.8% (14/26) adenoma and hyperplastic polyp tissues, and in 87.0% (60/69), 61.8% (21/34) and 42.3% (11/26) of corresponding fecal samples, respectively. In contrast, no methylated *SFRP2* gene was detected in mucosal tissues of normal controls, while two cases of matched fecal samples from normal controls were detected with hypermethylated *SFRP2*. A significant decrease ($P < 0.001$) in the rate of hypermethylated *SFRP2* gene was detected in the postoperative (8.7%, 6/69) fecal samples as compared with the preoperative fecal samples (87%, 60/69) of CRC patients. Moreover, no significant associations were observed between *SFRP2* hypermethylation and clinicopathological features including sex, age, tumor stage, site, lymph node status and histological grade, *etc.*

CONCLUSION: Hypermethylation of *SFRP2* gene in fecal DNA is a novel molecular biomarker of CRC and carries a high potential for the remote detection of CRC and premalignant lesions as noninvasive screening method.

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Key words: Colorectal cancer; Secreted frizzled-related protein 2; Feces; Methylation; Screening

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Wang DR, Tang D. Hypermethylated *SFRP2* gene in fecal DNA is a high potential biomarker for colorectal cancer noninvasive screening. *World J Gastroenterol* 2008; 14(4): 524-531 Available from: URL: http://www.wjgnet.com/1007-9327/14/524.asp DOI: http://dx.doi.org/10.3748/wjg.14.524

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide, and is the second major cause of death from cancer in Europe and in the USA[1,2]. Worldwide almost one million new cases occur annually, amounting to 492 000 related deaths[3]. However, the incidence and mortality of CRC have been decreasing over the last decade, and this may be attributable to more effective screening and surveillance[2]. CRC is most effectively treated when diagnosed at an early stage, but the early stage of CRC is mostly symptomless as well as delitescence nature. Approximately 40% of colorectal cancers are diagnosed with localized disease, which have approximately a 90% five-year survival rate after curative surgery. However, the prognosis worsens with advancing stage, and only 5% of patients diagnosed with distant metastases survive 5 years[4]. With the potential of both reducing mortality from CRC as well as enhancing primary prevention through detection and removal of lesions that could potentially develop into cancer, early detection of CRC will increase survival the most[5]. Therefore, the need for early detection is clear, and an effective screening test would have substantial clinical benefits.

A promising noninvasive screening tool for colorectal neoplasia detection is to assay for molecular biomarkers that represent a "specific" or "spectrum" of genetic and/or epigenetic alterations existing in gastrointestinal tumor cells shed into stool[6]. Potentially, both premalignant adenomas and CRC may be detected this way. Epigenetic alterations are defined as heritable signatures of information other than nucleotide sequences. The interest in this field (especially DNA methylation) is immense, and the past several years have seen an explosion in investigations based on the unique differences in methylation patterns between the cancerous and normal-appearing tissues, and this new assay facilitates its use for noninvasive CRC screening[7,8]. However, in contrast to the relatively well accepted mutation-based fecal DNA testing[9], methylation-based testing has been initiated more recently, and is beginning to identify adequate specific markers that are representative of epigenetic "signature" alterations. Methylation analysis of a number of gene promoters in DNA from stool samples has been less comprehensively investigated, but has been suggested to be a sensitive diagnostic tool for colorectal tumor[10-14].

Aberrant Wnt pathway signaling is an early progression event in up to 90% of CRCs[15]. Suzuki *et al*[16-18] have identified frequent promoter hypermethylation and gene silencing of the secreted frizzled-related protein (*SFRP*) genes in CRC, in which *SFRP2* gene at human chromosome 4q31.3 is claimed as a tumor suppressor gene inactivated by the epigenetic CpG hypermethylation in CRC[19]. *SFRP2* is split in three exons of variable size separated by two introns, and have dense CpG islands around its first exons[16]. The CpG islands were closed correlated with DNA methylation of tumor cells. Its expression reportedly is inhibited in CRC specimens by hypermethylation of a CpG island extending from the gene's 5'-flanking region through the first exon[18]. In CRC, *SFRP2* genes have been identified that are commonly unmethylated and expressed in normal colon mucosa but are methylated and silenced in CRC[16-18,20]. Detection of hypermethylation of *SFRP2* genes in human DNA isolated from stools might provide a novel strategy for the detection and investigation of CRC.

In the present study, we analyzed methylation of *SFRP2* in stools taken from CRC patients preoperatively and postoperatively, and from patients with benign colorectal diseases as well as from normal controls, and then evaluated the potential of fecal *SFRP2* gene hypermethylation as a non-invasive screening tool for CRC.

MATERIALS AND METHODS

Patients and collection of tissue and fecal DNA samples

Colorectal tissue samples were routinely collected from 69 patients with sporadic CRC, 60 patients with benign colorectal diseases (34 adenomas and 26 hyperplastic polyps) and 30 macroscopically normal subjects undergoing surgery and endoscopy at the First Affiliated Hospital of Yangzhou university from March 2005 to February 2007. Meanwhile, the matched fecal samples were collected from all CRC patients preoperatively at the time bowel preparation and at the 9th postoperative day, from the patients with benign colorectal diseases and from the endoscopically normal patients, and were kept at 4℃ until being processed. Within 12 h after collection, the samples were washed with 1 × PBS and centrifuged at 1800 r/min for 15 min to pellet the solid stool. The pellet of stool and tissue samples were all stored at -80℃. None of the patients had received chemotherapy or radiation therapy prior to surgery. All patients gave informed consent for their participation in the study which had been approved by the Ethical Committee of our university.

DNA isolation from tissue and fecal samples

Samples were randomly coded before processing to ensure adequate blinding of the clinical information. DNA was isolated from colonic tissues (5-10 mg) by using QIAamp DNA Mini Kit (Qiagen) and from stool samples (250 mg) by using QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol. HE-stained sections from tissue samples were examined by an experienced pathologist to confirm the histological diagnosis.

Bisulfite modification

The DNA was chemically modified by sodium bisulphate to convert all unmethylated cytosines to uracils while leaving methylcytosines unaltered (EpiTect® Bisulfite Kit, Qiagen), and eluted in 50 μL of elution buffer.

Methylation analysis

The bisulfite-modified DNA was used as a template for fluorescence-based real-time PCR (MethyLight) as described previously[21,22]. *SFRP2* gene was examined, and template-free distilled water was included as a negative control for amplification. The sequences of primers reported previously are listed in Table 1[14,23].

For the MethyLight, 3 μL of bisulfite-converted DNA was used in each amplification. PCR was performed in a reaction volume of 25 μL consisting of 17.875 μL of ddH_2O, 2.5 μL of 10 × PCR buffer, 2 μL of dNTP mixture, 0.25 μL of forward primer, 0.25 μL of reverse primer, 2 μL of template, and 0.125 μL of TaKaRa Taq HS (Hot Start) at the following conditions: pre-denaturation at 95℃ for 5 min, followed by 40 amplification cycles of denaturation at 95℃ for 30 s, primer annealing at 50℃ for 30 s (U primer), and extension at 62℃ for 30 s (M primer), and finally a further extension at 72℃ for 5 min. The stain was SYBR green I and PCR was performed in the LightCycle Fluorescent Quantitation PCR Detection System (LightCycler 1.0, Switzerland). A standard curve was created by plotting the logarithmic amount of the standard universal methylated DNA (31.25 pg to 10 ng) against the threshold cycle (CT) value.

Statistical analysis

To compare characteristics of the different groups of patients and biologic samples, two sided Student's *t* test, Chi-square test, and Fisher exact test were used as appropriate. Data were analyzed using SPSS10.0 software. $P < 0.05$ was considered statistically significant.

Table 1 *SFRP2* gene primer sequences, annealing temperature and product size for MethyLight assays

CpG status	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (℃)	Product size (bp)
M	GGGTCGGAGTTTTTCGGAGTTGCGC	CCGCTCTCTTCGCTAAATACGACTCG	62	138
U	TTTTGGGTTGGAGTTTTTTGGAGTTGTGT	AACCCACTCTCTTCACTAAATACAACTCA	50	145

M: Methylated; U: Unmethylated.

Table 2 Hypermethylation of *SFRP2* in tissue and stool specimens taken from the same patients with colorectal cancer, adenoma, polyp and normal controls

Characteristics	Case (*n*)	Tissue M1 (%)	χ^2	*P*	Fecal M2 (%)	χ^2	*P*	Sensitivity M2/*n*	Specificity
Colorectal Cancer	69	63 (91.3)	75.326[1]	0.000[1]	60 (87.0)	57.588[1]	0.000[1]	87.00%	
Age									
< 50	20	17 (85.0)	1.410	0.235	16 (80.0)	1.202	0.273		
⩾ 50	49	46 (93.9)			44 (89.8)				
Sex									
Male	37	34 (91.9)	0.035	0.852	32 (86.5)	0.016	0.901		
Female	32	29 (90.6)			28 (87.5)				
TNM stage									
Ⅰ / Ⅱ	30	27 (90.0)	0.114	0.736	25 (83.3)	0.614	0.433		
Ⅲ / Ⅳ	39	36 (92.3)			35 (89.7)				
Lymph node status									
Positive	27	25 (92.6)	0.093	0.761	24 (88.9)	0.146	0.702		
Negative	42	38 (90.5)			36 (85.7)				
Infiltration									
Mucosa underlayer	21	18 (85.7)	1.188	0.276	17 (81.0)	0.959	0.327		
Muscular coat	48	45 (93.8)			43 (89.6)				
Tumor site									
Rectum	30	27 (90.0)	0.306	0.858	27 (90.0)	0.428	0.786		
Left hemicolon	18	17 (94.4)			15 (83.3)				
Right hemicolon	21	19 (90.5)			18 (85.7)				
Adenoma	34	27 (79.4)	27.579[1]	0.000[1]	21 (61.8)	21.016[1]	0.000[1]	61.80%	
Tubular adenoma	11	8 (72.7)	0.518	0.772	6 (54.5)	0.563	0.755		
Villous adenoma	10	8 (80.0)			6 (60.0)				
Tubulovillous adenoma	13	11 (84.6)			9 (69.2)				
Hyperplastic polyp	26	14 (53.8)	21.538[1]	0.000[1]	11 (42.3)	9.926[1]	0.002[1]	42.30%	76.80%
Normal control	30	0			2 (6.7)				

M1: Hypermethylated SFRP2 in tissue; M2: Hypermethylated SFRP2 in stool. [1]Represents the value of χ^2 and *P vs* normal controls. There were significant differences in prevalence of hypermethylated SFRP2 in tissue and stool between cancer and adenoma (χ^2 = 2.921, *P* = 0.087 and χ^2 = 8.606, *P* = 0.003, respectively) and between cancer and hyperplastic polyp (χ^2 = 17.253, *P* < 0.001 and χ^2 = 19.936, *P* < 0.001, respectively). However, a significant difference was observed in prevalence of hypermethylated SFRP2 between adenoma and hyperplastic polyp only in tissue (χ^2 = 4.450, *P* = 0.035) but not in fecal specimens (χ^2 = 2.241, *P* = 0.134).

RESULTS

Hypermethylation of SFRP2 gene in hyperplastic polyps, advanced adenomas and colorectal cancers

We examined hypermethylation of *SFRP2* gene in DNA from 69 patients with CRC, 34 with advanced adenoma (characterized by size ⩾ 1 cm, villous histology, and high-grade dysplasia) and 26 with hyperplastic polyps. *SFRP2* was hypermethylated in 91.3% (63/69), 79.4% (27/34) and 53.8% (14/26) of the CRCs, advanced adenomas and hyperplastic polyps, respectively. In the colorectal advanced adenomas, the prevalence of hypermethylated *SFRP2* was substantially higher in the tubulovillous and villous adenomas as compared with the tubular adenomas, and was much more in the adenomas accompanying with epithelium dysplasia. The prevalence of hypermethylated *SFRP2* had a gradually increased tendency from hyperplastic polyps to CRCs. In contrast, none of the normal colorectal mucosa of endoscopically normal patients showed any methylated bands (Figures 1-3, Table 2).

The hypermethylation of *SFRP2* gene was significantly different between cancer and normal mucosa ($P < 0.001$), between advanced adenoma and normal mucosa ($P < 0.001$), between hyperplastic polyp and normal mucosa ($P < 0.001$), between cancer and hyperplastic polyp ($P < 0.001$), and between adenoma and hyperplastic polyp ($P = 0.035$); however, no significant difference was found between cancer and adenoma ($P = 0.087$) (Table 2).

Detection of SFRP2 hypermethylation in fecal DNA

Following the performance of the MethyLight assays on the DNA extracted from the tissue samples, we next assessed matched fecal DNA from the corresponding patients to detect *SFRP2* hypermethylation in fecal DNA from individuals with CRC or premalignant lesions. We found that 92.1% (58/63) of CRCs, 66.7% (18/27) of advanced adenomas and 71.4% (10/14) of hyperplastic polyps had concordant hypermethylated *SFRP2* in their tissue DNA and fecal DNA, respectively. Two patients with CRC, 3 with advanced adenoma and 1 with hyperplastic polyp had methylated *SFRP2* only in the fecal DNA, while 5 patients with CRC, 9 with advanced adenoma and 4 with

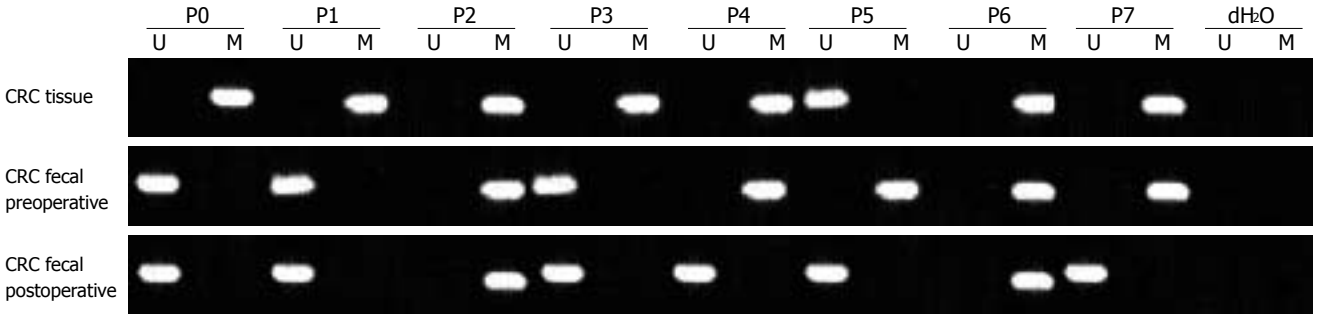


Figure 1 Methylation status of *SFRP2* gene in preoperative and postoperative fecal specimens and in CRC tissues taken from the same patients. P: Patient; M: Methylated; U: Unmethylated.

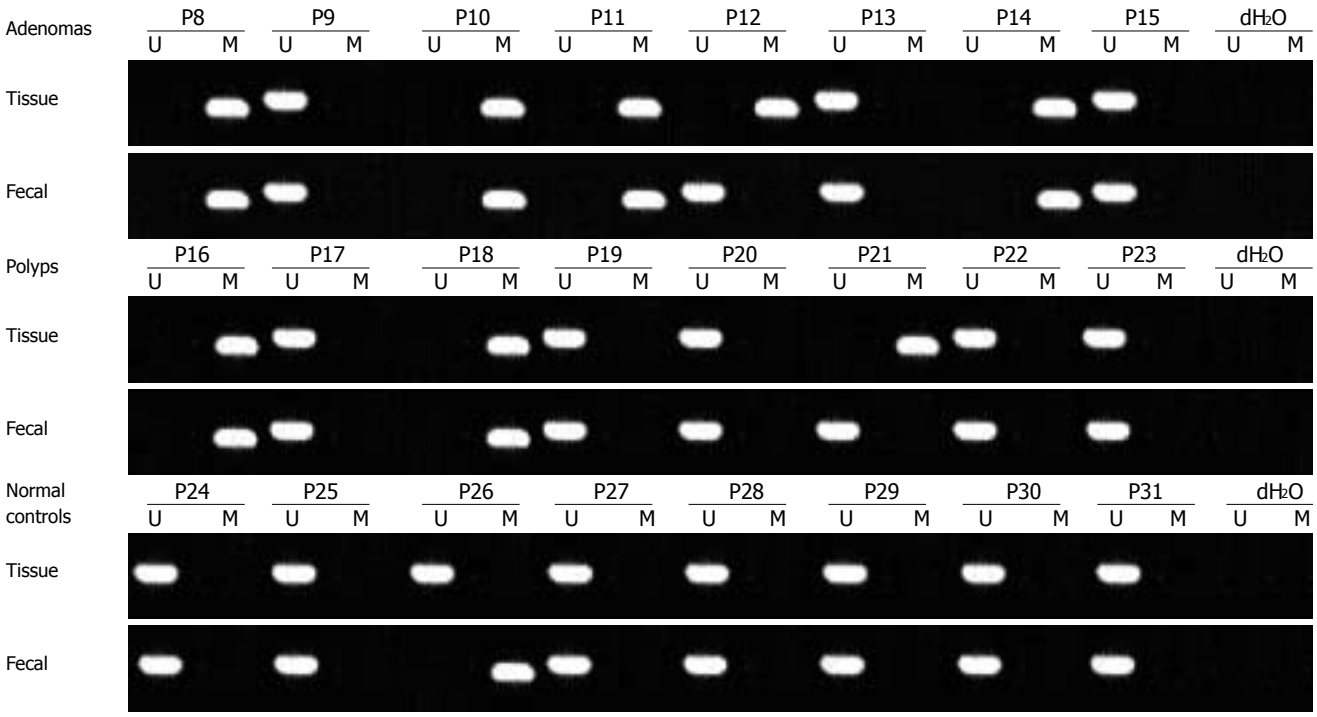


Figure 2 Methylation status of *SFRP2* gene in tissue and feces taken from the same patients with benign colorectal diseases and normal controls.

hyperplastic polyp had methylated *SFRP2* only in the tissue DNA. All of the remaining subjects that had unmethylated *SFRP2* in the tissue DNA showed unmethylated *SFRP2* in their fecal samples (Figures 1-4, Table 2).

Comparison of the performance characteristics of *SFRP2* MethyLight assays showed that the assays could detect 92.1%, 66.7% and 71.4% of individuals with CRC, advanced adenoma and hyperplastic polyp, respectively, that carried hypermethylated *SFRP2*. The clinical sensitivities of *SFRP2* hypermethylation in fecal DNA for detecting the presence of CRC, advanced adenoma and hyperplastic polyp were 87.0% (60/69), 61.8% (21/34) and 42.3% (11/26), respectively. To evaluate the clinical specificity of this assay, we next analyzed fecal DNA of 30 normal control individuals, and found that only 2 (6.7%) samples were positive for hypermethylation of *SFRP2* (Table 2).

Detection of SFRP2 hypermethylation in fecal DNA of CRC patients preoperatively and postoperatively

We compared the *SFRP2* hypermethylation in fecal DNA collected preoperatively and at the 9th postoperative day from CRC patients. Five of the 60 CRC patients whose stool carried hypermethylated *SFRP2* preoperatively had detectable hypermethylated *SFRP2* in their fecal samples postoperatively, showing a significant decrease postoperatively ($P < 0.001$). Only one of the nine CRC patients whose stool carried unmethylated *SFRP2* preoperatively had detectable hypermethylated *SFRP2* in the fecal sample postoperatively. All of the remaining 8 CRC patients with fecal unmethylated *SFRP2* preoperatively showed fecal unmethylated *SFRP2* postoperatively.

Moreover, no significant associations were observed between *SFRP2* gene hypermethylation and clinicopathological features, including sex, age, tumor stage, site, lymph node status and histological grade, etc (Table 2).

DISCUSSION

CRC is a common malignancy that arises from benign

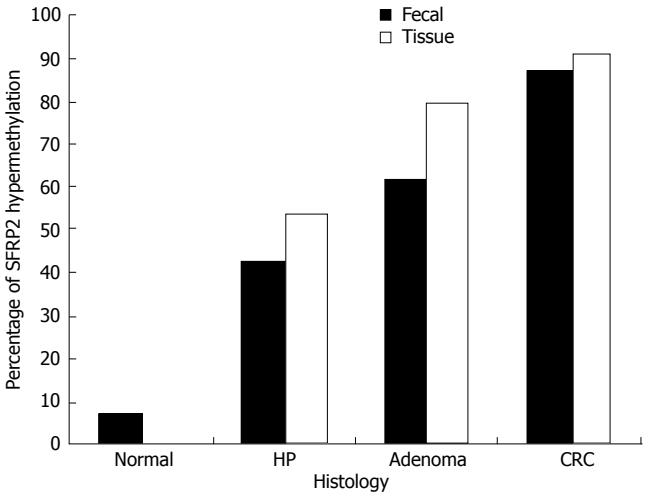


Figure 3 Prevalence of hypermethylated SFRP2 in stool and tissue specimens taken from the subjects with normal colonic mucosa, hyperplastic polyps (HP), adenomas and colorectal cancers (CRCs).

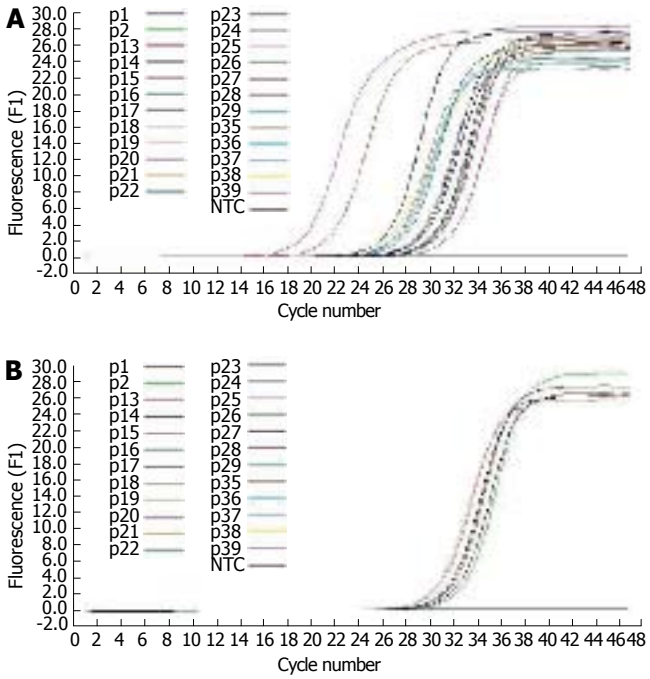


Figure 4 The photofluorogram of hypermethylated *SFRP2* gene in fecal specimen performed by MethyLight. **A**: Photofluorogram of *SFRP2* gene performed in M (methylation) primer and its amplification condition; **B**: Photofluorogram of *SFRP2* gene performed in U (unmethylation) primer and its amplification condition. P represents the patients; and NTC represents the negative controls.

neoplasms and evolves into adenocarcinomas through a stepwise histological progression sequence, proceeding from either adenomas or hyperplastic polyps/serrated adenomas[24-26]. Genetic alterations have been associated with specific steps in this adenoma-carcinoma sequence and are believed to drive the histological progression of CRC[27]. Recently, epigenetic alterations, especially DNA methylation, have been shown to occur in colorectal polyps, adenomas and CRCs[28-30]. The aberrant methylation of genes appears to act together with genetic alterations to drive the initiation and progression of colorectal polyp and adenoma to CRC[31]. Genetic alterations occur during the adenoma-carcinoma sequence of colorectal cancer formation and drive the initiation and progression of colorectal cancer formation. The aberrant methylation of genes is an alternate, epigenetic mechanism for silencing tumor suppressor genes in colorectal cancer. Thus, the aberrant methylation of genes appears to increase most significantly during the progression of early adenomas to advanced adenomas, and the frequency of specific gene methylation at the different steps of the adenoma-carcinoma progression sequence varies in a gene-specific fashion[28]. DNA methylation changes have been recognized as a key mechanism of colorectal carcinogenesis. Because of the ubiquity of DNA methylation changes and the ability to detect methylated DNA in stool, analysis of aberrantly methylation in stool DNA might provide a novel strategy for noninvasive detection of CRC[7,32-34]. Due to the heterogeneity of tumors, usually multiple markers distributed throughout the human genome need to be analyzed, and this is labor-intensive and does not allow for high throughput screening. Therefore, markers with high sensitivity and good specificity are needed[34]. *SFRP2* is one of the soluble modulators of a putative tumorigenic pathway-the Wnt signaling pathway, and is frequently hypermethylated in colorectal cancer, adenoma and aberrant crypt foci (ACF)[16-18,20,35]. At present, there are only a few researches in literature regarding *SFRP2* hypermethylation in CRC tissue and in stool of CRC patients[14,20,33]. In this study, we assessed the methylation status of *SFRP2* in tissue and matched stool samples from patients with CRC with respect to a series of healthy individuals and patients with benign colorectal diseases, using fluorescence-based real-time PCR assay (MethyLight). Our results suggested *SFRP2* hypermethylation as a highly sensitive and specific independent marker for the fecal screening of CRC.

It has been shown that hypermethylation of *SFRP2* genes, inhibitors of Wnt signaling pathway[16-18,20], is a common early event in the evolution of colorectal tumor, occurring frequently in ACF, hyperplastic polyp and adenoma, which is regarded as the earliest lesion of multistage colorectal carcinogenesis. It appears that hypermethylation of *SFRP2* gene might serve as indicator for early CRC[10,14,33]. We detected hypermethylation of *SFRP2* in tissue and matched fecal samples from the corresponding patients with CRC and premalignant diseases. The results showed that *SFRP2* was hypermethylated in 91.3%, 79.4% and 53.8% of the CRCs, advanced adenomas and hyperplastic polyps, respectively, thereby indicating that hypermethylation of *SFRP2* occurs in hyperplastic polyps and becomes more frequent in advanced adenomas and then in colorectal cancers. Moreover, the clinical sensitivities for detecting the presence of CRC, advanced adenoma and hyperplastic polyp were 87.0%, 61.8% and 42.3%, respectively, and the overall specificity was 76.8%. In addition, there was a trend, although not statistically significant, of *SFRP2* hypermethylation more among the adenomas accompany with epithelium dysplasia. On contrary, hypermethylation of *SFRP2* gene was found in stool of two endoscopically

normal subjects, which might be most likely owing to the *SFRP2* methylation occurring frequently in premalignant aberrant crypt foci (ACF) that is overlooked at colonoscopy[14,16-18]. Polyp and adenoma are the early events of colorectal tumorigenesis, and have a high risk of malignant transformation[28,36]. Our results show that hypermethylated SFRP was detectable not only in stools of patients with CRC, but also in stools of patients with premalignant lesions, such as advanced adenomas, suggesting that hypermethylated SFRP is a sensitive fecal-based molecular marker for screening CRC and premalignant lesions.

At present, there are only two institutions which had studied the methylated *SFRP2* in stool as a molecule marker for CRC screening: Müller *et al*[10] proposed *SFRP2* hypermethylation as a sensitive marker which is able to detect 77%-90% of CRCs; and Huang *et al*[14,33] reported that methylated *SFRP2* occurred in 94.2%, 52.4%, 37.5%, and 16.7% of patients with CRC, adenoma, hyperplastic polyp, and ulcerative colitis, respectively. In this study, we simultaneously detected hypermethylated *SFRP2* in tissue and fecal samples taken from the patients with CRC and benign colorectal diseases and from the healthy individuals. When compared with the two aforementioned researches, our results showed that hypermethylated *SFRP2* in stool had almost the same high sensitivity for detecting CRC, but had significantly higher detection rate for hyperplastic polyp and advanced adenoma. With the same ethnic groups, the discrepancy in performance of our study and that of Huang *et al*[14,33] is likely attributable to the more matched fecal and tissue samples of the same patients, but the use of a more sensitive methylation assay, MethyLight[21,37-39], may also account for the higher sensitivity and lower specificity. Despite of that, the high level of *SFRP2* hypermethylation in patients with hyperplastic polyps is somewhat surprising. There is little evidence that the hyperplastic polyps, which are usually not thought to be premalignant, with methylated DNA would develop into CRC; therefore the hyperplastic polyps will be counted as false positive when using *SFRP2* hypermethylation-based screening strategy. Whether hypermethylation of *SFRP2* gene can really identify this non-malignant diseases is still unclear. As DNA methylation is more common in advanced adenomas and CRC[40], all our findings and that of Huang *et al*[14,33] collectively suggest that *SFRP2* hypermethylation may reflect the malignant potential of these lesions. Because of the nature of CRC evolution (hyperplastic polyp -serrated adenoma-carcinoma sequence), the levels of hypermethylated *SFRP2* in stool of patients with hyperplastic polyps may have substantial clinical benefits through the detection and endoscopic removal of this benign disease, which needs large sample to further study. In short, patients with fecal hypermethylated *SFRP2* may belong to high-risk individuals and need more accurate clinical examinations and follow-up.

Furthermore, we compared the hypermethylated *SFRP2* gene in stool of CRC patients preoperatively and at the 9th postoperative day. The results indicated that the prevalence of hypermethylated *SFRP2* in stool of CRC patients decreased significantly postoperatively, which showed a new evidence that the hypermethylated *SFRP2* gene in stool comes from the CRC tissues and *SFRP2* hypermethylation in stool carries a high potential for the remote detection of CRC as a noninvasive screening method.

At present, there are several methods for detecting of CRC and premalignant lesions[41,42], but none of them is really suitable for screening CRC. Fecal occult blood testing has been shown to reduce CRC-related mortality and is cheap but has low sensitivity. Colonoscopy, with a high sensitivity and specificity for detection of CRC and large adenomas, requires a thorough bowel preparation, causes discomfort and small but non-negligible risk of major complications in patients, and is an invasive procedure. CT colonography[43], also known as virtual colonoscopy, looks promising but so far has been performed unevenly in the hands of community radiologists. However, aberrant methylation in fecal DNA as a molecular marker has developed a new way for screening CRC and premalignant lesions. Our study demonstrated initially that hypermethylated *SFRP2* gene in stool is a promising and noninvasive sensitive marker for screening colorectal neoplasia.

ACKNOWLEDGMENTS

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COMMENTS

Background

Silence of secreted frizzled-related protein 2 (*SFRPs*) genes, which are secreted glycoproteins working as inhibitory modulators of the Wnt signaling pathway induced by promoter hypermethylation, plays a key role in colorectal tumorigenesis, and hypermethylation of *SFRPs* genes is a common early event in the evolution of colorectal cancer (CRC). *SFRP2* is one of the *SFRPs* gene family members and is frequently hypermethylated in ACF, adenoma and colorectal cancer. Thus, detection of hypermethylation of *SFRPs* genes in human DNA isolated from stools might provide a novel strategy for the detection and investigation of CRC.

Research frontiers

Epigenetic hypermethylation can result in transcriptional silencing of tumor suppressor genes and is considered to be a key event of sporadic CRC, and gradually become the important areas in the research of CRC. Detection of hypermethylation of *SFRPs* genes in human DNA isolated from stools might provide a novel strategy for the detection and investigation of CRC.

Innovations and breakthroughs

A more sensitive methylation assay (MethyLight) was performed to analyze *SFRP2* gene promoter methylation status in a blinded fashion in tumor tissues and in matched stool samples from the same patients with CRC and colorectal benign diseases. Furthermore, we compared the hypermethylated *SFRP2* gene in stool of patients with CRC preoperatively and at the 9th postoperative day.

Applications

Our study demonstrated initially that hypermethylated *SFRP2* gene in stool is a promising and noninvasive sensitive single marker for screening colorectal neoplasia.

Terminology

Wnt signaling pathway: Signals are transduced by Wnt ligands through frizzled (Fz) membrane receptors by competition with Fzs for Wnt ligands or by direct formation of nonsignaling complexes with Fzs themselves. Secreted frizzled-related proteins (SFRPs): These are secreted proteins of about 36 ku that receive their name by

their structural homology with the extracellular cystein-rich domain (CRD) of frizzled (Fz), a family of developmentally important signaling molecules, and have the ability to bind to both Wnt and Fz proteins. Epigenetic mechanism: Somatic cells acquire changes in gene expression that are transmissible through mitosis but which do not involve any alterations to the DNA sequence. CpG island DNA methylation: The aberrant DNA methylation affects CpG-rich regions, called "CpG islands", in the 5' region of genes and results in transcriptional silencing through complex effects on transcription factor binding and associated changes in chromatin structure.

Peer review

This paper studied the use of hypermethylation of *SFRP2* promoter as a means for diagnosing colorectal cancer. The authors showed that, compared to individuals with normal colon, patients with colon cancer, advanced adenoma and hyperplastic polyps had a high prevalence of *SFRP2* hypermethylation in the tissues and stools. There also appeared to be a significant difference in the methylation patterns in patients with colon cancer before and after surgery. They conclude that hypermethylation of *SFRP2* may be a sensitive diagnostic test for colorectal neoplasm.

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COLORECTAL CANCER

An effective vaccine against colon cancer in mice: Use of recombinant adenovirus interleukin-12 transduced dendritic cells

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Abstract

AIM: To investigate the effect of a vaccine with recombinant adenovirus interleukin-12 (AdVIL-12) transduced dendritic cells (DCs) against colon cancer in mice.

METHODS: DCs and AdVIL-12 were incubated together at different time intervals and at different doses. Supernatant was collected and tested for IL-12 by enzyme-linked immunosorbent assay (ELISA). In order to determine whether tumor cell lysate-pulsed (TP) AdVIL-12/DCs enhance therapeutic potential in the established tumor model, CT26 colon tumor cells were implanted subcutaneously (s.c.) in the midflank of naïve BALB/c mice. Tumor-bearing mice were injected with a vaccination of CT26 TP AdVIL-12/DCs on d 3 and 10. As a protective colon tumor model, naïve BALB/c mice were immunized s.c. in their abdomens with CT26 TP AdVIL-12/DCs twice at seven day intervals. After the immunization on d 7, the mice were challenged with a lethal dose of CT26 tumor cells and survival times were evaluated. Subsequently, cytotoxic T lymphocyte (CTL) activity and interferon gamma (IFNγ) secretion was evaluated in the immunized mice, and assayed CTL *ex vivo*.

RESULTS: Murine DCs were retrovirally transduced with AdVIL-12 efficiency, and the AdVIL-12 transduced DCs secreted a high level of IL-12 (AdVIL-12/DCs, 615.27 ± 42.3 pg/mL *vs* DCs, 46.32 ± 7.29 pg/mL, $P < 0.05$). Vaccination with CT26 TP AdVIL-12/DCs could enhance anti-tumor immunity against CT26 colon tumor in murine therapeutic models (tumor volume on d 19: CT26 TP AdVIL-12/DCs 107 ± 42 mm^3 *vs* CT26 TP DCs 383 ± 65 mm^3, $P < 0.05$) and protective models. Moreover, the CT26 TP AdVIL-12/DC vaccination enhances tumor-specific CTL activity, producing high levels of IFNγ in immunized mice. *Ex vivo* primed T cells with AdVIL-12/DCs were able to induce more effective CTL activity than in primed T cells with CT26 TP/DCs (E:T = 100:1, 69.49% ± 6.11% specific lysis *vs* 37.44% ± 4.32% specific lysis, $P < 0.05$).

CONCLUSION: Vaccination with recombinant AdVIL-12 transduced DC pulsed tumor cell lysate enhance anti-tumor immunity specific to colon cancer in mice.

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Key words: Vaccination; Dendritic cells; Cytokine; Interleukin-12; Colon cancer; Immunotherapy

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INTRODUCTION

Colon cancer is one of the most common cancers[1]. Although there has been great progress in colon cancer therapy, it is still difficult to treat advanced colon cancer because it has usually metastasized to lymph glands and other organs. Currently, radical surgery represents the standard method of therapy. Adjuvant therapy such as chemotherapy and radiation therapy have been widely applied, but colon cancer control at the advanced stage remains difficult[2,3]. A new adjuvant therapeutic method is needed in order to improve the 5-year survival rate of patients with colon cancer. Today, tumor immunotherapy for colon cancer is extremely appealing[4,5].

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that both initiate and modulate the immune response[6]. DCs are cells in the pathway of antigen capture and presentation to T cells, with the

unique ability to directly prime naïve $CD4^+$ and $CD8^+$ T cells. They posses the ability to efficiently uptake, process, and present antigens on major histocompatibility complex (MHC) class Ⅰ and Ⅱ molecules, together with co-stimulatory molecules such as B7 and CD40[7]. Therefore, DCs are believed to be essential for stimulating tumor-specific cytotoxic T lymphocytes (CTL) and inducing the protective and therapeutic anti-tumor immunity against cancer cells. There have been attempts to produce vaccines based on DCs, including immunizations by DCs pulsed with tumor lysates or apoptotic tumor cells or tumor RNA. DCs have been fused with tumor cells, transduced with peptides to elicit specific antitumor T cells and enhance anti-tumor immunity[8]. Now, DCs can be pulsed with synthetic peptides or proteins derived from known tumor associated antigens (TAA), such as MUC1, Her-2/neu, tyrosinase, CEA or Melan-A/MART. Primary clinical studies were performed on melanoma patients using DCs pulsed with peptides or loaded with tumor cell lysates[9]. This data revealed that such vaccines could be safely applied without significant side effects and induce antigen-specific CTL responses *in vivo*.

Interleukin (IL)-12 is a heterodimeric, proinflammatory cytokine produced by macrophages, DCs, and B cells, which stimulates T lymphocytes and natural killer cells to release interferon gamma (IFNγ) and promote an anti-tumor response[10,11]. Some studies have shown that IL-12 has powerful adjuvant properties and the immune-modulating characteristics of IL-12 are considered to be responsible for these effects. Therapeutic activity with IL-12 has been observed in various murine tumor models[12,13]. Transduction of vaccine cells with IL-12 genes can substantially improve their immunogenicity[10]. Trials with IL-12 gene-modified melanoma cells have also been initiated in humans[14]. Saika T *et al*[15] reported that DCs engineered to express murine IL-12 and pulsed with a tumor lysate, induce specific T cell responses and anti-tumor immunity against a mouse prostate cancer model. This work suggests that adenovirus-mediated IL-12 gene transduction enhanced the immune responses.

In this study, we demonstrated that vaccination with DCs engineered to express IL-12 by adenovirus-mediated gene transfer and pulsed with CT26 colon tumor cell lysate, enhanced tumor-specific CTL activity and anti-tumor immunity in CT26 colon tumor models, suggesting promising strategy for colon cancer immunotherapy.

MATERIALS AND METHODS

Animals

Female BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) mice (8-10 wk old) were purchased from the Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). All mice were kept under pathogen-free conditions in the animal center of the Shanghai Second Medical University (Shanghai, China).

Cell

CT26 ($H\text{-}2^b$), a mouse colon adenocarcinoma cell line, and SGC-7901 ($H\text{-}2^b$), the gastric cancer cell line were purchased from the Shanghai Cell Biology Institutes, Chinese Academy of Sciences (Shanghai, China). CT26 cell lines grow in BALB/c mice. Both cell lines were cultured in RPMI (Roswell Park Memorial Institute) medium 1640 (GIBCO, USA) containing 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L glutamine, penicillin G (100 U/mL), and streptomycin (100 μg/mL) at 37℃ in a humidified incubator supplemented with 5% CO_2.

Major

Murine granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, IL-2, and IL-7 were purchased from Becton Dickinson (New Jersey, USA). Phenotypic analysis, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies against murine cell surface molecules, such as MHC Ⅰ ($H\text{-}2K^d$), MHC Ⅱ ($I\text{-}A^b$), CD11b, CD11c, CD40, CD80, CD86, and CD8α, were provided by Pharmigen (CA, USA). Mitomycin C (MMC) was purchased by Jingmei Biothe (Shenzhen, China).

Generation of bone marrow-derived DCs

Primary bone marrow DCs (BM-DCs) was obtained from mouse bone marrow precursor according to a protocol by Zhang *et al*[16]. BALB/c mice bone marrow was obtained from tibia and femurs by flushing with the media. Tissue was minced in a single-cell suspension through a nylon mesh. BALB/c mice bone marrow cells were stained sequentially with biotinylated anti-c-kit MAb and Streptavidm MicroBeads (Dynal, Norway). $c\text{-}kit^+$ hematopoietic progenitor cells were magnetically isolated with a MiniMACS separator (Milteyi Biotec, Auburn, CA) from the of BALB/c mice bone marrow cells. The cells (6×10^6) were cultured for 7 d in fresh RPMI medium 1640 containing 10% FCS, GM-CSF (4 ng/mL), and IL-4 (10 ng/mL).

Immunofluorescence staining

DC immunofluorescence analysis was performed as previously described[16,17]. In brief, DCs prepared and isolated from BALB/c mice, as described above (2×10^5-4×10^5 cells), were incubated with FITC-labeled MAbs against CD40, CD11b, CD11c, or CD80 and PE-labeled MAbs against MHC Ⅰ, MHC Ⅱ, CD86 or CD8α followed by FACScan analysis (Becton Dickinson, USA). Instrument compensation was set in each experiment using two-color stained samples.

Recombinant adenovirus vector

Recombinant adenoviruses IL-12 (AdVIL-12) ecoding the murine IL-12 gene were donated by Dr. Y. Y. Zhang (Health Science Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China). These recombinant adenoviruses were propagated in 293 cells and purified on a CsCl density gradient. Their titres were determined using a plaque assay on 293 cells. Aliquots of the adenovirus solutions were stored at -80℃.

Adenoviral transduction of DCs and secretion of IL-12

DCs generated on d 8 were plated at 2×10^6 cells/well in 1ml of RPMI 1640 medium containing 10% FCS with AdVIL-12 at a multiplicity of infection (MOI) of 100. After 2 h incubation at 37℃ in a humidified incubator

supplemented with 5% CO_2 with gentle agitation every 20 min, the DCs culture medium was replaced with 2 mL of RPMI 1640 medium supplemented with 10% FCS, and the cells were incubated for another 48 h. AdVIL-12 transduced DCs (AdVIL-12/DCs) were harvested, washed twice with PBS, and used for the tumor cell lysate pulsing *in vitro*. Immunofluorescence analysis of AdVIL-12/DCs was performed as described above.

DCs (1×10^6) were infected with AdVIL-12 at MOI 100. Viral supernatant was replaced after 2 h with the cell culture medium. The supernatant was collected and tested for mIL-12 by enzyme-linked immunosorbent assay (ELISA) kit (Rockford, IL, USA) at different time intervals (0, 12, 24, 48, 72, 96 h). Moreover, DCs were infected with AdVIL-12 at different MOI (50, 100, 200, 300, 500). The supernatant was collected 48 h later and tested for mIL-12 by ELISA.

Pulsing DCs with tumor cell lysates

DCs were pulsed with freeze-thawed tumor lysate at a 1:3 DCs: tumor ratio. CT26 tumor (6×10^6) were lysed by rapid freezing (liquid nitrogen) and thawing in a 37℃ bath three times. The AdVIL-12 transduced DCs (AdVIL-12/DCs, 2×10^6 cells/mL) or bone marrow-derived DCs (2×10^6 cells/mL) were incubated in six-well plates in the presence of CT26 tumor lysates (6×10^6 cell equivalents/mL) in RPMI Medium 1640 containing 10% FCS, GM-CSF (4 ng/mL), and IL-4 (10 ng/mL) for one day at 37℃ and 5% CO_2. These CT26 tumor cell lysate-pulsed (TP) AdVIL-12/DCs or DCs were used for vaccination after one day.

Tumor model and DC-based vaccination

In the established tumor model, CT26 tumor cells (5×10^5 cells per mouse) were implanted subcutaneously (s.c.) in the midflank of naïve BALB/c mice (10/each group). Tumor-bearing mice were injected with 1×10^6 DCs vaccination: CT26 TP AdVIL-12/DCs or CT26 TP DCs on d 3 and 10. As a positive control, tumor bearing mice were treated in parallel with unstimulated DCs, CT26 tumor lysate alone, or PBS alone. Tumor growth was assessed by calipers (mean of 2 perpendicular diameters) every 2-3 d. Tumor volume was estimated by the formula $4/3\ \pi r^3$.

In the protective anti-tumor experiment, each group consisting of 10 naïve BALB/c mice were injected s.c. in their abdominal walls with 1×10^6 DCs vaccination: CT26 TP AdVIL-12/DCs or CT26 TP DCs on d 0, 7, and 14. Control mice were injected with unstimulated DCs, CT26 tumor lysate alone, and PBS alone. Each mouse was challenged s.c. with a lethal dose of 2×10^6 CT26 cells on d 21. Survival differences among the groups receiving different vaccinations were evaluated following the primary challenge with CT26 cells.

Assays for cytotoxic T lymphocyte activity and interferon gamma secretion in the immunized mice

To confirm that tumor-specific cytotoxic T lymphocytes (CTLs) had been generated in the immunized mice, splenic $CD3^+$ T cells from tumor-free mice that survived the CT26 tumor challenge through d 61 following DC vaccination, were magnetically isolated using CD3 MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany). T cells (1×10^6 cells) from surviving mice were restimulated *ex vivo* by culturing in IMDM containing 10% FCS and MMC-treated CT26 tumor cells (1×10^5). Five days later, the MMC-treated CT26 tumor cells and the T cells were collected for measuring CTL activity and IFNγ secretion. The restimulated effector T cells (2×10^5 in 100 μL per well) were added to the wells containing target CT26 or SGC-7901 tumor cells (5×10^4 in 100 μL per well) in 96-well plates. Supernatant from each well was collected after 20 h and was measured cytolytic activity against target CT26 tumor cells and SGC-7901 tumor cells with a Cytotoxicity Detection Kit (LDH; Boehringer Mannheim, Mannheim, Germany), IFNγ production was determined with the mouse IFNγ ELISA kit (Endogen, Woburn, MA, USA). In some experiments, the target CT26 tumor cells (1.5×10^5) were incubated with a MAb to MHC class I molecules (anti-H2Db/H2Kb; 50 g/mL) or with control antibody (anti-H2Dd; 50 g/mL) at 37℃ for 30 min before the addition of restimulated effector T cells to evaluate the specificity of CTL activity.

Assays for cytotoxic T lymphocyte activity ex vivo

To confirm that tumor-specific cytotoxic T lymphocytes (CTLs) can be generated ex vivo, splenic $CD3^+$ T cells (1×10^6 cells/mL) were magnetically isolated from naïve BALB/c mice using CD3 microBeads. These cells were cultured in RPMI medium 1640 containing 10% FCS, then primed *ex vivo* in the presence of cytokines including IL-2 and IL-7 (5 ng/mL per) at d 0, 7, and 14 with CT26 TP AdVIL-12/DCs or CT26 TP DCs at a 1:20 stimulator to responder cell ratio. Unpulsed DCs and AdVIL-12/DCs were used as controls. Fresh medium containing IL-2 and IL-7 was exchanged every four days. The primed T cells were effector cells, CT26 or SGC-7901 tumor cells were target cells. On d 21, target cell suspension was added into 96 well plates, and effector cells were titrated to the dilutions target cells by serial dilutions (E-T mix, E:T, 1:1, 5:1, 10:1, 20:1, 50:1, 100:1). Supernatant from each well was collected after 20 h in order to measured cytolytic activity against target CT26 and SGC-7901 tumor cells, which was done with a cytotoxicity detection kit as mentioned above.

Statistical analysis

Differences were evaluated using Statistical Package for Social Science 11.0 (SPSS11.0). Statistical analysis was performed using a Student's *t* test. Survival differences among mice groups were evaluated with a log-rank test of the Laplan-Meier survival curves. Statistical tests were two-tailed. P values < 0.05 were considered to be statistically significant.

RESULTS

Phenotypic markers of DC analysis by FACScan

The phenotypic profile of a representative population of bone marrow DCs was first determined. These DCs were expressed in moderate levels of MHC class Ⅰ, CD11b and CD11c, high levels of MHC class Ⅱ, moderate levels of

Table 1 Phenotypic characteristics of AdVIL-12/DCs

DCs[1]	Mean fluorescence intensity							
	MHC Ⅰ	MHC Ⅱ	CD40	CD11b	CD11c	CD80	CD86	CD8α
Nontransduced	35	92	19	48	26	112	69	10
AdVIL-12	49	304[a]	23	56	30	271[a]	229[a]	9

[1]Nontransduced DCs and AdVIL-12 transduced DCs were harvested 6 d after the culture initiation and stained with the listed antibodies. Background intensities = 4-6. [a]$P < 0.05$ *vs* those of nontransduced DCs.

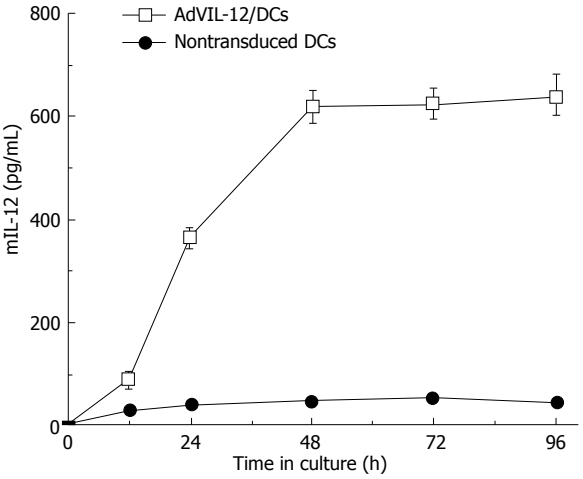


Figure 1 IL-12 production by the DCs transduced with AdVIL-12. DCs and AdVIL-12 were incubated together at different time intervals (0 h, 12 h, 24 h, 48 h, 72 h, 96 h). Supernatant was collected and tested for mIL-12 by ELISA, with nontransduced DCs as control. Data is representative of two independent experiments, with a given as mean ± SE.

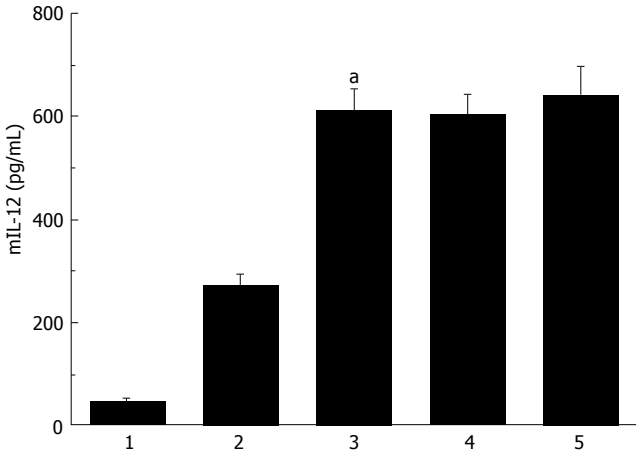


Figure 2 IL-12 production by the DCs transduced with AdVIL-12. BM-DCs was transduced with AdVIL-12 at various doses. DCs supernatant was examined 48 hrs later for the IL-12 production by ELISA, with nontransduced DCs was as control. Statistical analysis used the paired Student's *t* test. Data are given as mean ± SE. [a]$P < 0.05$ AdVIL-12/DCs (MOI 100) compared with nontransduced DCs or AdVIL-12/DCs (MOI 50). 1: Nontransduced DCs. 2: AdVIL-12/DCs (MOI 50). 3: AdVIL-12/DCs (MOI 100). 4: AdVIL-12/DCs (MOI 300). 5: AdVIL-12/DCs (MOI 500).

the co-stimulatory molecules CD40, CD80, and CD86, and very low levels of CD8α. Phenotypic changes within the DCs induced by AdVIL-12 transduction were examined by phenotypic analysis. Expression levels of CD80, CD86, and MHC class Ⅱ were increased in AdVIL-12/DCs compared with the DCs (Table 1).

Secretion of IL-12by transduced DCs in vitro

Secretion analysis of AdVIL-12/DCs, DCs and AdVIL-12 was determined by incubation at different cultured time intervals and at different MOI, and supernatant was collected and tested for mIL-12 by ELISA. Figure 1 illustrated that AdVIL-12/DCs secrete high levels of IL-12 at 48 h intervals. However, the levels of IL-12 did not increase with time. In addition, the DCs transfected with AdVIL-12 at a MOI of 100 showed a significant increase in the IL-12 level against the control DCs. This was statistically significant (AdVIL-12/DCs, 615.27 ± 42.3 pg/mL *vs* DCs, 46.32 ± 7.29 pg/mL, [a]$P < 0.05$, Figure 2).

Vaccination with CT26 TP AdVIL-12/DCs enhances anti-tumor immunity in therapeutic or protective models

In order to determine whether or not CT26 TP AdVIL-12/DCs enhance therapeutic potential in the established tumor model, CT26 tumor cells (1×10^6) were implanted s.c. in the midflank of naïve BALB/c mice (10/each group). Tumor-bearing mice were injected with different vaccinations on d 3 and 10. Figure 3 shows that tumor growth was significantly inhibited in mice vaccinated with CT26 TP AdVIL-12/DCs. Vaccination with CT26 TP AdVIL-12/DCs significantly retarded tumor growth, and exerted a greater anti-tumor effect than CT26 TP DCs (tumor volume on d 19: PBS control 856 ± 95 mm^3, CT26 tumor lysate control 748 ± 67 mm^3, unstimulated DCs control 813 ± 73 mm^3, CT26 TP DCs 383 ± 65 mm^3, and CT26 TP AdVIL-12/DCs 107 ± 42 mm^3, CT26 TP AdVIL-12/DCs *vs* CT26 TP DCs, [a]$P < 0.05$). Differences between CT26 TP AdVIL-12/DCs and CT26 TP DCs were statistically significant.

CT26 TP AdVIL-12/DCs were used in vaccinations to see whether there were increases in anti-tumor immunity. Naïve BALB/c mice were immunized s.c. twice at 7 d intervals in abdominal walls with CT26 TP AdVIL-12/DCs, CT26 TP/DCs, unpulsed DCs, tumor lysates alone, and PBS, respectively. On d 7 after secondary immunization, these mice were challenged with 2×10^6 CT26 tumor cells. All mice vaccinated with unstimulated DCs, tumor lysates alone, and PBS died within 30 d after CT26 tumor cell challenge (Figure 4). However, mice immunized with CT26 TP AdVIL-12/DCs had significantly prolonged survival compared with the control or other vaccination groups. During the 60-d observation period, the mice vaccinated with CT26 TP AdVIL-12/DCs had a 90% survival rate and the mice

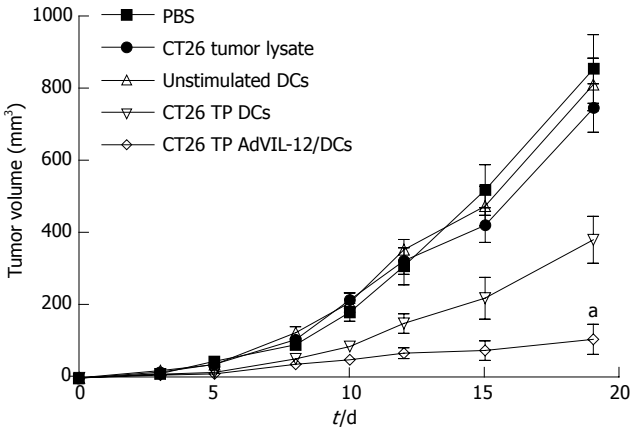


Figure 3 Vaccination with CT26 TP AdVIL-12/DCs enhances anti-tumor immunity in therapeutic tumor models. CT26 tumor cells (5×10^5 cells per mouse) were implanted s.c. in the midflank of naïve BALB/c mice. On d 3 and 10, tumor-bearing mice were injected with 1×10^6 DCs vaccination with CT26 TP AdVIL-12/DCs. As control, tumor bearing mice were treated in parallel with CT26 TP DCs, unstimulated DCs, CT26 tumor lysate alone, or PBS alone. Tumor growth was assessed by calipers every 2-3 d thereafter. Tumor volume was estimated by the formula $4/3\ \pi r^3$. Statistical analysis used the paired Student's *t* test. Data are given as mean ± SE (n = 10 mice in each group). $^aP < 0.05$, CT26 TP AdVIL-12/DCs *vs* CT26 TP DCs or the other control groups (d 19).

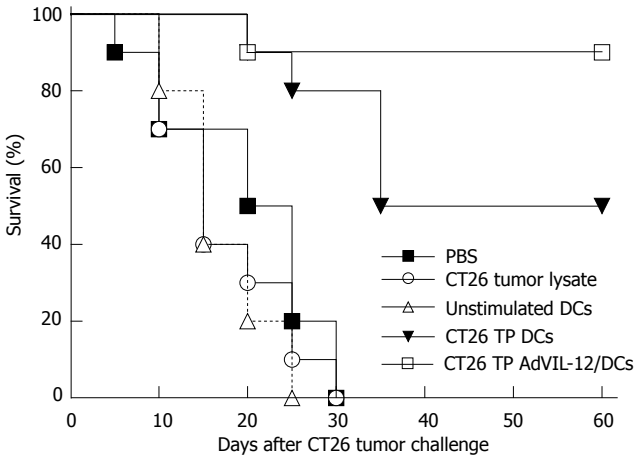


Figure 4 Protective effect of CT26 TP AdVIL-12/DCs in CT26 tumor models. Naïve BALB/c mice were injected s.c. in their abdominal walls on d 0, 7, and 14 with 1×10^6 DCs vaccination: CT26 TP AdVIL-12/DCs or CT26 TP DCs. Mice injected with unstimulated DCs, CT26 tumor lysate alone, and PBS alone were used as controls. On d 21, each mouse was challenged s.c. with a lethal dose of 2×10^6 CT26 cells. Survival was observed after the challenge with tumor cells. Survival rate was compared with a log-rank test of Kaplan-Meier curves. The survival advantage conferred by the CT26 TP AdVIL-12/DC was statistically significant compared with CT26 TP DCs group and other control groups (Kaplan-Meier, $P < 0.05$).

vaccinated with CT26 TP/DCs had a 50% survival rate, respectively, compared with the mice vaccinated with unpulsed DCs (0%), tumor lysates alone (0%), and PBS (0%). Survival advantage of CT26 TP AdVIL-12/DC was statistically significant compared with the CT26 TP DC group or other control groups (Kaplan-Meier, $P < 0.05$).

Enhancement of tumor-specific CTL responses

In order to determine whether vaccination with CT26 TP AdVIL-12/DCs enhances tumor-specific CTL responses and to see if the response was tumor specific, we isolated splentic $CD3^+$ T cells obtained from mice still alive 60 d (in CT26 TP AdVIL-12/DCs or CT26 TP/DCs group) after the CT26 tumor cells challenge. Assayed levels of IFNγ were determined, which is a reflection of immune activation and cytotoxic activity against CT26 tumor cells or SGC-7901 tumor cells. Results demonstrated that the splenic mice $CD3^+$ T cells vaccinated with CT26 TP AdVIL-12/DCs produce higher levels of IFNγ in vitro when stimulated with CT26 tumor cells than T cells obtained from mice vaccinated with CT26 TP/DCs (1685.88 ± 61.54 pg/mL in CT26 TP AdVIL-12/DCs group *vs* 912.58 ± 38.33 pg/mL in CT26 TP DCs, $P < 0.05$, Figure 5A). At the same time they showed substantially enhanced cytotoxic activity (21.86% ± 1.29% specific lysis in CT26 TP AdVIL-12/DCs group *vs* 12.67% ± 0.76% specific lysis in CT26 TP DCs, $P < 0.05$, Figure 5B). In addition, all splenic $CD3^+$ T cells obtained from mice vaccinated with CT26 TP AdVIL-12/DCs and vaccinated with CT26 TP DCs produced high levels of IFNγ and showed specific cytotoxic activity against CT26 tumor cells, but no cytotoxic activity when stimulated with SGC-7901 tumor cells (Figure 5A and B). Furthermore, antibodies to MHC class I molecules abrogated the cytolytic activity against CT26 tumor cells. Vaccination with CT26 TP AdVIL-12/DCs was shown to induce a MHC-I restricted CTL response (Figure 5C).

Finally, vaccination with CT26 TP AdVIL-12/DCs enhances tumor-specific CTL responses *ex vivo*. Naïve mouse splenic T cells were primed ex vivo with CT26 TP AdVIL-12/DCs or CT26 TP/DCs in the presence of IL-2 and IL-7 to elicit cytolytic reactivity against tumor cells. Unpulsed DCs and AdVIL-12/DCs were used as controls. Results demonstrated that primed T cells *in vitro* with AdVIL-12/DCs or CT26 TP/DCs were able to lyse parental CT26 tumor cells specifically, but not SGC-7901 tumor cells. Also, T cells primed with unpulsed DCs or AdVIL-12/DCs did not induce specific CTL against CT26 tumor cells or SGC-7901 tumor cells (Figure 6A and B). Furthermore primed T cells with AdVIL-12/DCs are able to induce effective CTL activity more than primed T cells with CT26 TP/DCs (E:T = 100:1, 69.49% ± 6.11% specific lysis in CT26 TP AdVIL-12/DCs group *vs* 37.44% ± 4.32% specific lysis in CT26 TP DCs, $^aP < 0.05$, Figure 6A). It is possible that a vaccination with CT26 TP AdVIL-12/DCs may enhance the anti-tumor immune response to syngeneic tumor cells.

Together, these results suggest that mice vaccinated with CT26 TP AdVIL-12/DCs, and subjected to CT26 tumor cells, and primed T cells *in vitro* with AdVIL-12/DCs, were more effective in generating generated MHC class I restricted CTLs specific for CT26 tumor cells.

DISCUSSION

In our experiments we showed that murine BM-DCs could be retrovirally transduced with AdVIL-12 efficiency, and the AdVIL-12 transduced DCs could secrete high levels of IL-12. Vaccination with DCs that were engineered to express IL-12 by adenovirus-mediated gene transfer could

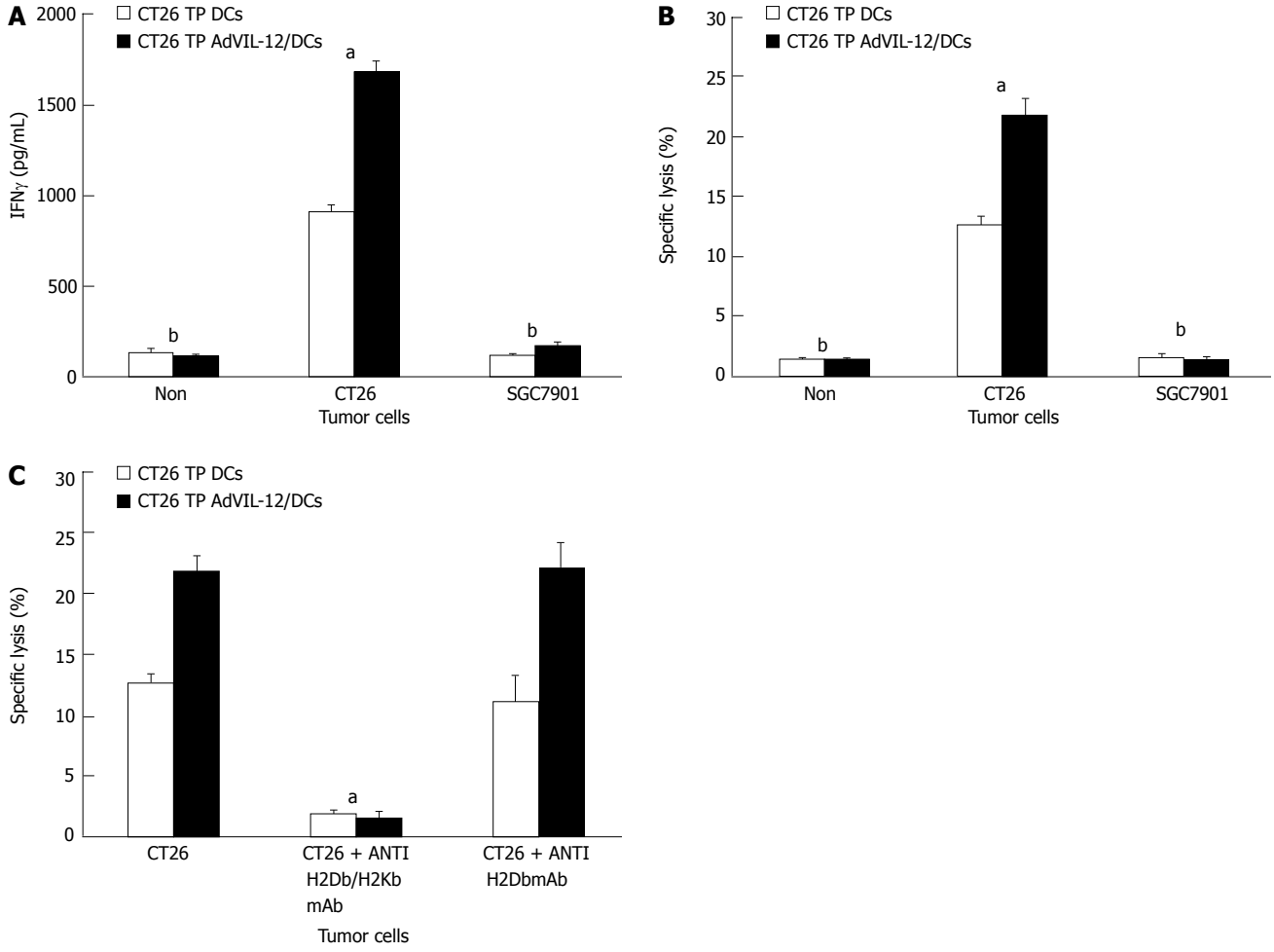


Figure 5 Assays for IFNγ secretion and tumor-specific CTL activity in the immunized mice. Splenic $CD3^+$ T cells from mice that survived the CT26 tumor challenge until d 61 in protective models were magnetically isolated. $CD3^+$ T cells were restimulated *ex vivo* by culturing with the MMC-treated CT26 tumor cells. Then these restimulated effector T cells were added to the wells containing target CT26 or SGC-7901 tumor cells in 96-well plates. After 20 h, supernatant from each well was collected for measuring IFNγ production with the mouse IFNγ ELISA kit (**A**), and for measuring cytolytic activity with a Cytotoxicity Detection Kit (**B**). In some experiments, the target CT26 tumor cells were incubated with a MAb to MHC class I molecules (anti-H2Db/H2Kb) or with control antibody (anti-H2Dd) before the addition of restimulated effector T cells to evaluate the specificity of CTL activity. Statistical analysis used the paired Student's *t* test. Data are given as mean ± SE. A, B $^aP < 0.05$, CT26 TP AdVIL-12/DCs compared with CT26 TP DCs in the CT26 groups, $^bP < 0.01$ CT26 *vs* SGC7901 or Non, respectively. (**C**), $^aP < 0.05$, CT26 + antiH-2kb/DbmAb *vs* CT26 or CT26 + antiH-2DbmAb, respectively.

enhance anti-tumor immunity against CT26 colon tumor in murine therapeutic and protective models. Moreover, in CTL response, vaccination with CT26 TP AdVIL-12/DCs enhanced tumor-specific CTL activity to produced high levels of IFNγ.

Successful genetic modification of murine BM-DCs with retroviral vectors was demonstrated. Previous reports illustrated the feasibility of retroviral gene modification of human DCs by tansduction of $CD34^+$ progenitor cells with the subsequent addition of cytokine to promote differentiation and maturation[18,19]. In this study murine DCs were transduced using cocultivation with retroviral producer cells. The murine BM-DCs transduced with AdVIL-12 at different MOI or different time intervals in culture. DCs transduced with AdVIL-12 at an MOI of 100 shown limited toxicity and maximal production of IL-12, and at 48 h intervals in culture, demonstrate maximal production of IL-12. Phenotypes of DCs retrovirally transduced with IL-12 were evaluated. The experiments demonstrated expression levels of CD80, CD86, and MHC Ⅱ were enhanced in AdVIL-12/DCs compared with the non-transduced DCs, without apparent surface molecules changes. These phenotypic changes could be due in part to the direct effect of IL-12 or, more likely, indirectly through IFNγ production by contaminating T cells or natural killer cells. Grohmann U *et al*[20] reported that IL-12 acts directly on DCs to promote nuclear localization of nuclear factor *k*B and primes DCs for IL-12 production. Suzuki T *et al*[21] also reported that the DCs transduced AdVIL-12 showed increased levels of the costimulatory molecules (CD80 and CD86), MHC Ⅱ molecules, compared with the non-transduced DCs. These changes could explain, in part, why IL-12-transduced DCs are more effective in tumor treatment application.

Tumor immunity induction can be initiated by the effectors of innate immunity and can be further developed by cells of adaptive immunity, with DCs playing a central regulatory role. Several steps are involved including (a) recognition of tumor molecules by DC precursors, (b)

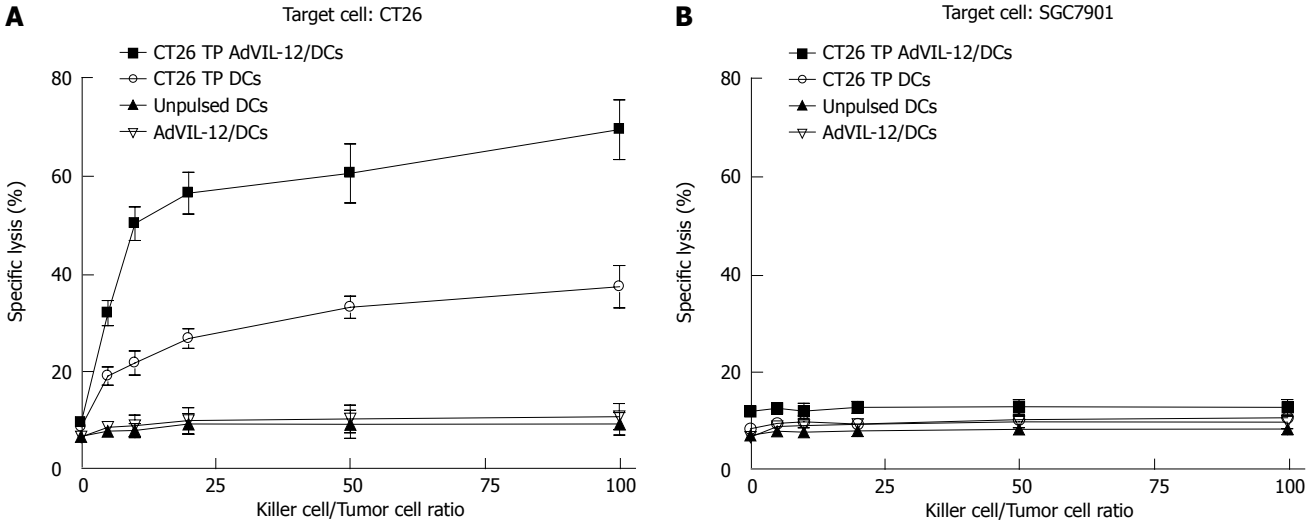


Figure 6 Generation of tumor-specific cytotoxic T cells *ex vivo*. Splenic $CD3^+$ T cells were magnetically isolated from naïve BALB/c mice. These cells were cultured *ex vivo* in the presence of cytokines including IL-2 and IL-7 and with CT26 TP AdVIL-12/DCs or CT26 TP DCs at a stimulator to responder cell ratio of 1:20. The primed T cells were effector cells, CT26 or SGC-7901 tumor cells were target cells. Then these effector T cells were titrated by serial dilution (E-T mix, E:T 1:1, 5:1, 10:1, 25:1, 50:1, 100:1), and their lytic activity against CT26 or SGC-7901 were assayed by a Cytotoxicity Detection Kit. Statistical analysis used the paired Student's *t* test. Data are given as mean ± SE. A $^aP < 0.05$, CT26 TP AdVIL-12/DCs *vs* CT26 TP DCs or other control groups in the CT26 groups.

direct and IFN-γ-mediated killing of transformed cells by NK/NK T cells activated by DCs, (c) capture and cross-presentation of released-tumor-associated Ags (TAAs) by immature DCs, (d) selection and activation of TAA-specific T cells, as well as nonspecific effectors including macrophages and eosinophils, and (e) homing of TAA-specific T cells to the tumor site and recognition elements leading to the elimination of tumor cells[22]. DCs-based vaccination had showed efficiently anti-tumor effective in many tumor models and in the clinical studies. Kono K *et al*[23] reported that tumor vaccination therapy with DCs pulsed with HER-2/neu-peptides may be a potential candidate for the novel treatment of gastric cancer patients. Tumor markers (CEA or CA19-9) were decreased after vaccination in 3 out of 9 patients. Two patients had more than 50% tumor regression, and two presented a mixed response. These studies revealed that such vaccines could be safely applied without significant side effects and induced antigen-specific CTL responses *in vivo*.

Cytokine gene therapy is becoming a promising weapon against cancer[24]. Adenovrial-based cytokine gene therapy has many advantages over other forms of cytokine delivery[25]. Adenoviral vectors allow local, high-efficiency, but transient transgene expression, generating high-level but self-limited cytokine production in treated tumors. Adenoviral vectors are transduction targets in a heterogeneously growing population of tumor cells. Recombinant IL-12 has generated interest as a potential cancer therapeutic agent due to the ability of murine IL-12 to induce tumor regression and to cure in various mouse models of cancer. Studies examining the anti-tumor mechanisms of IL-12 have implicated various cellular mediators including CD8 T cells, NK cells, and NK T cells[26,27]. Taking full advantages of characters of DCs and IL-12 respectively, constitutive production of IL-12 by AdVIL-12/DCs, coupled with their ability to take up and process tumor antigens, migrate to tumor site or lymph nodes can induce an effective immune response. In this study, a DCs-based vaccination transduced with AdVIL-12 was successfully produced using cocultivation methods.

In the experiment *in vivo*, significant anti-tumor effects were observed in therapeutic models or protective models when vaccination with CT26 TP AdVIL-12 transduced DCs were injected. These anti-tumor effects were not observed, however, when nontransduced DCs were injected. Complete tumor eradication was observed in 30%-50% of mice treated with a single injection of CT26 TP AdVIL-12/DCs in therapeutic models. In protective models, the mice immunized with CT26 TP AdVIL-12/DCs had significant survival rates. During the 60-d observation period, mice vaccinated with CT26 TP AdVIL-12/DCs showed 90% survival rate, compared to mice vaccinated with CT26 TP/DCs (50%). These results suggest that overexpression of IL-12 by DCs in the peripheral blood, the tumor site, or in the secondary lymphoid sites, alternatively, could be important for an anti-tumor response in addition to the phenotypic changes in AdVIL-12/DCs. Chemokine (IP-10 and Mig)[28,29] production induced by IL-12, is responsible for these effects, at least in part, through enhanced recruitment of cytolytic effector cells to develop into tumors, plus possibly antiangiogenic effects (Fas/FasL)[30].

Systemic immune responses, as demonstrated by CTL activity and IFNγ production *in vivo* and *ex vivo*, were also significantly higher and tumor specific when AdVIL-12/DCs were used. High cytolytic activity in association with a TH1-type response could possibly contribute to the profound anti-tumor effects that we observed. In contrast, responses induced by nontransduced DCs were far less potent. Tatsumi T *et al*[31] reported that the anti-tumor effect of DCs-based vaccination is dependent on the production of the TH1-type immunostimulatory cytokine such as IFNγ, IL-12. Therefore, the level of IFNγ

production as a result of IL-12 may play an important role in the increased anti-tumor immune responses.

In summary, research data demonstrates that vaccination with recombinant adenoviruses interleukin-12 transduced dendritic cells pulsed tumor cell lysate enhance anti-tumor immunity specific to colon cancer in mice. This approach is superior to that observed by nontransduced DCs. These results suggest that an evaluation of AdVIL-12/DCs in colon cancer patients with is an important next step. A clinical trial evaluating this approach is currently in preparation.

COMMENTS

Background

Dendritic cells (DCs) play a central role in immune responses and may be useful adjuvants for tumor vaccination. Heterodimeric cytokine interleukin (IL)-12 is the key cytokine mediating the generation of a TH1-type cytokine expression pattern in both T lymphocytes and NK cells, resulting in cytotoxic cellular immune responses. IL-12 has been shown to generate powerful anti-tumor responses in many tumor models. IL-12 production by recombinant adenoviruses IL-12 (AdVIL-12) transduced DCs, coupled with their ability to take up and process tumor antigens, induce an effective anti-tumor immune response.

Research frontiers

DCs are believed to be essential for stimulating tumor-specific CTL and inducing the protective and therapeutic anti-tumor immunity against cancer cells. Attempts to produce vaccines based on DCs have been developed including immunizations by DCs pulsed with tumor lysates or apoptotic tumor cells or tumor RNA, fused with tumor cells, transduced with peptides. Now, DCs can be pulsed with synthetic peptides or proteins derived from known tumor associated antigens (TAA) such as MUC1, Her-2/neu, tyrosinase, CEA or Melan-A/MART. Vaccines based on DCs have been safely applied in clinic. Some studies have shown that IL-12 has powerful adjuvant properties and immune-modulating characteristics of IL-12 are considered to be responsible for such adjuvant effects. Therapeutic activity of IL-12 has been observed in various murine tumor models. Transduction of vaccine cells with IL-12 genes can substantially improve their immunogenicity. Trials with IL-12 gene-modified melanoma cells have also been started in humans.

Innovations and breakthroughs

Taking full advantages of characters of DCs and IL-12 respectively, a DCs-based vaccination transduced with AdVIL-12 was successfully produced using cocultivation methods. Vaccination with DC engineered to express IL-12 by adenovirus-mediated gene transfer could enhance anti-tumor immunity in murine therapeutic and protective models. Vaccination can be application for immunotherapy against colon cancer in mice successfully.

Applications

Vaccination with AdVIL-12 transduced DCs pulsed tumor cell lysate enhance anti-tumor immunity specific to colon cancer in mice. These results suggest that an evaluation of AdVIL-12/DCs in colon cancer patients with is an important next step. A clinical application of this approach is currently in preparation.

Terminology

DCs (Dendritic cells), bone marrow DCs (BM-DCs), antigen presenting cells (APCs), Interleukin-12 (IL-12), recombinant adenoviruses interleukin-12 (AdVIL-12), AdVIL-12 transduced DCs (AdVIL-12/DCs), major histocompatibility complex (MHC), cytotoxic T lymphocyte (CTL), interferon gamma (IFNγ),

Peer review

The authors examined that murine BM-DCs could be retrovirally transduced with AdVIL-12 efficiency, and the AdVIL-12 transduced DCs could be secret high levels of IL-12. Vaccination with DC engineered to express IL-12 by adenovirus-mediated gene transfer could enhance anti-tumor immunity against CT26 colon tumor in murine therapeutic and protective models. Moreover, in CTL response vaccination with CT26 TP AdVIL-12/DCs enhanced tumor-specific CTL activity, to produced high levels of IFNγ.

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BASIC RESEARCH

Influence of kavain on hepatic ultrastructure

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Abstract

AIM: To investigate whether the major kavalactone kavain imposes adverse effects on the liver ultrastructure and function by affecting vascular and microvascular architecture and altering hepatocellular morphology.

METHODS: Kavain solution (10 μg/mL or 43.5 μmol/L) was perfused for 2 h in isolated rat livers. After standard fixation and tissue preparation, the samples were examined by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and light microscopy (LM).

RESULTS: LM, SEM, and TEM examinations indicated kavain-treated rat livers (n = 4) displayed severe vascular and endothelial damage compared to control livers (n = 4).

CONCLUSION: The data so far support the hypothesis that kavain induces adverse effects on liver; additional investigations with other kavalactones and their effects on liver are urgently needed.

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Key words: Kava; Kavalactones; Kavain; Liver; Electron microscopy

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INTRODUCTION

Kava-kava, or kava, (*Piper methysticum* Forst.) is a South Pacific plant that has been used by indigenous people as an intoxicating traditional beverage since ancient times[1-4]. Various preparations of kava have been marketed since the 1980s, especially in Europe and North America to manage mild anxiety, tension, and restlessness. Some reports also indicate that kava preparations may have analgesic, spasmolytic, neuroprotective, and antimitotic activities[5-8]. However, a number of case reports have raised serious concerns about kava's safety. These reports suggest that, occasionally, even normal doses (200-300 mg/d standardized to contain 70% kavalactones) of kava can cause severe liver injury[9-13]. On the basis of these reports, regulatory agencies have banned or restricted sale of kava and kava products in many countries.

The main active constituents believed to be responsible for the pharmacological (and perhaps toxicological) effects of *Piper methysticum* are the styryl α-pyrones (called kavapyrones or kavalactones). To date, 18 kavalactones have been identified, among these, six major kavalactones constitute approximately 95% of the lipid extract derived from the dried roots and rhizomes; these are kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin. Figure 1 shows kavain's chemical structure, its molecular formula is $C_{14}H_{14}O_3$, and molecular weight is 230[14-16].

The aim of this study was to investigate whether the major kavalactone, kavain, induces ultrastructural changes and function of the liver instead of biochemical perturbations. This was achieved by examining the vascular, microvascular, and hepatocellular morphological changes that might occur after perfusion of rat livers with kavain. This hypothesis was tested as there are literature evidence that kava might produce general adverse effects on the liver.

MATERIALS AND METHODS

Materials

Kavain (purity 95%) was purchased from ChromaDex, California, USA. Bovine serum albumin (BSA, Fraction V), taurocholic acid, and other chemicals were analytical grade and purchased from Sigma (Sydney, Australia).

Animals

Male Sprague-Dawley rats (Gore Hill, NSW Australia) of about 200 grams were used; prior to the experiments the animals were housed in a 12 h light-dark cycle and

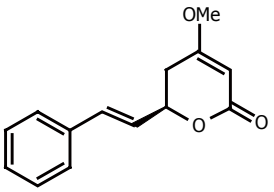


Figure 1 Chemical structure of kavain (KA).

controlled temperature environment with free access to standard laboratory chow and water. The study was approved by the Animal Ethics Committee of The University of Sydney.

Isolated rat liver perfusions with kavain

Rat livers were perfused with 10 μg/mL (43.5 μmol/L) kavain in 100 mL perfusate (1% BSA and 0.1% glucose in Krebs-Henseleit bicarbonate buffer) for 120 min for the kavain treatment groups; livers were perfused only with 100 mL perfusate for 120 min in the control group. The surgical and perfusion techniques have been described previously[17]; briefly this involved *in situ* cannulation of the portal vein and inferior vena cava under barbiturate anaesthesia and harvesting of the liver for recirculating perfusion. Liver viability during the perfusion was assessed by the pH gradient between inflow and outflow perfusate, bile flow rate and the gross appearance of the liver[18]. After 120 min of perfusion, the livers were perfusion fixed with 100 mL of 1.5% glutaraldehyde in 0.12 mol/L cacodylate buffer at pH 6.9 for 7 min. Following this the livers were stored in buffer at 4℃ until preparation for electron microscopic examination.

Liver tissue preparation for light and electron microscopy

Tissue samples were prepared according to standard protocols[19,20]. Briefly, glutaraldehyde perfused-fixed samples were cut into 1 mm^3 blocks in 1.5% glutaraldehyde/0.12 mol/L cacodylate buffer at pH 6.9. Blocks were then submerged in 1% osmium tetroxide/0.1 mol/L cacodylate buffer for 1 h at 4℃, gradually dehydrated in ethanol and embedded in epon. 50-80 nm ultra thin sections were cut, stained first with uranyl acetate and then with lead citrate. Slides were examined in a Philips Zeiss 902 transmission electron microscope at 80 kV.

Semi-thin (1000 nm) sections of TEM embedded liver samples were cut with a glass knife for overall light microscopic observations. Sections were stained with 1% toluidine blue solution prepared in 1% sodium tetraborate buffer, mounted in DePex (Cat. No. RT 13510, Electron Microscopy Science, Hatfield PA, USA) and examined in bright field mode and recorded using a digital CCD camera.

For scanning electron microscopy (SEM), liver tissue samples were prepared according to standard protocols[19,20]. Briefly, liver tissue was glutaraldehyde-prefixed, cut to 1 mm^3 samples and following glutaraldehyde fixation, tissue blocks were postfixed in 1% osmium tetroxide and dehydrated in graded ethanol solutions. The liver tissue was then dried with hexamethyldisilazane and subsequently broken in liquid nitrogen, mounted on stubs and sputter coated with a thin layer of 20 nm gold[21]. SEM-samples were examined in a Philips SEM 505 at 30 kV. Imaging and morphometric analysis was performed on randomly acquired digitized SEM images at magnifications of × 3000, × 5000 or × 20000 as previously described[22]. For light- and electron microscope studies all experiments were repeated at least four times.

RESULTS

Given that there are literature indications that kava affects liver function it was logical to examine with the aid of scanning electron microscopy, the influence of kavain on the surface liver architecture and vasculature in general and on the intactness of the endothelial lining in particular. Under control (no kavain) conditions sinusoids in the liver lobule show an intact endothelial lining, consisting of endothelium with flattened processes perforated by small pores. These pores (i.e., fenestrae) measure about 150-200 nm in diameter and are arranged in groups, also called sieve plates (Figure 2, SEM images). Following kavain treatment the liver tissue displays overall loss of architecture in general; the narrowing of blood vessels, constriction of sinusoidal blood vessels and retraction of the endothelium. Only few intact pores (fenestrae) can be observed since most of the pores are lost, and gaps form in endothelium which are from the dilatation of pores, these gaps can disturb normal liver transportation.

In order to investigate these changes in further detail at overall low versus high magnification light- (Figure 3, LM images) and transmission (Figure 4, TEM images) electron microscopy were applied on sectioned liver tissue, respectively. LM and TEM examination of control tissue shows an intact relationship between the sinusoidal liver vasculature and the neighbouring liver parenchymal cells; i.e., the sinusoid is patent and empty, the wall of the sinusoid is composed of a thin layer of porous (i.e., fenestrated) endothelium, which is covering the space of Disse, filled by microvilli extending from the parenchymal cell surface. These parenchymal cells contain glycogen, a few lipid vesicles, and numerous organelles in their cytoplasm. However, after 120 min of kavain treatment, the LM observations show loss of overall liver architecture; the closed, disrupted endothelium are confirmed, there are no signs of inflammatory reactions (such as platelets and white blood cells or other related cell types); the parenchymal cells of hepatocytes which are lying close to the sinusoidal vasculature are vacuolated, and the vascular bed is constricted, narrowed. The TEM results after kavain treatment confirm the SEM observations; the existence of gaps which indicate disruption of the normal liver architecture, only few pores in endothelial lining were found; and some strong vacuolized hepatocytes surround the blood vessels; finally vascular constriction can be noticed from the images, and it appears that the diameters of the blood vessels are smaller when compared to the control (no kavain) samples. In other words, after kavain treatment the normal hepatic vascular bed is lost. This may cause portal and central pressure perturbations and affect normal physiological liver function.

Noteworthy is that the severe changes in sinusoidal microvasculature and overall loss of the integrity of the liver parenchymal cell architecture was accompanied with striking structural alterations of the liver macrophage population

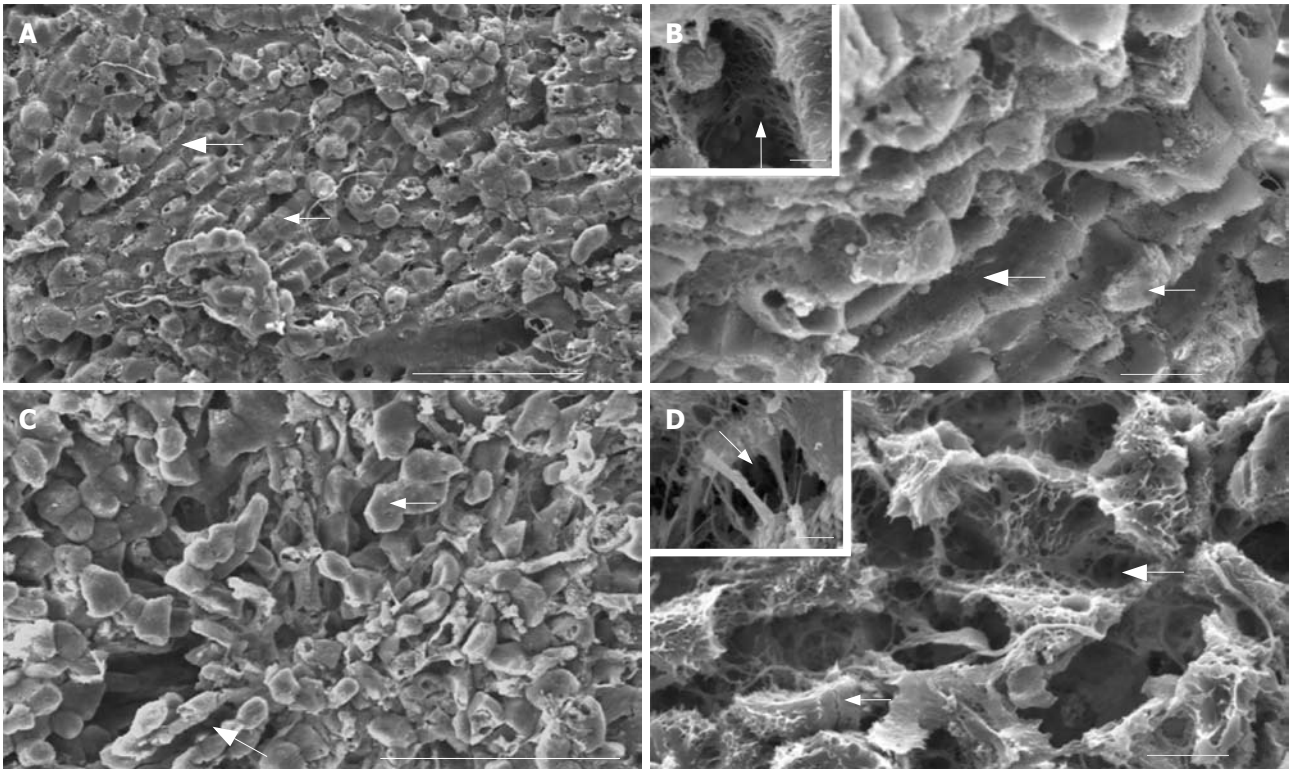


Figure 2 Scanning electron micrographs of control (**A** and **B**) and kavain-treated (**C** and **D**) livers. **A**: Overall low magnification image shows the intact histological relationship between the liver sinusoidal vascular bed (large arrow) and the neighbouring liver parenchymal cells (small arrow) of control liver tissue. Scale bar, 100 micrometer; **B**: Intermediate magnification of control liver tissue reveals the intact hepatic endothelial lining (large arrow) and its relationship with the neighbouring hepatocytes (small arrow). Scale bar, 40 micrometer. Inset depicts the pores or so-called fenestrae (arrow) at high magnification (bar represents 1 micrometer); **C**: Observation of kavain-treated liver tissue at low magnification reveals loss of the normal straight and parallel aligned sinusoidal blood vessels, instead contracted and curly microvessels can be seen (large arrow). Note that the hepatocytes appear to be swollen and have lost their relationship with the bordering endothelial lining (small arrow). Scale bar, 100 micrometer; **D**: When the same tissue is observed at higher magnification the endothelial damage in the form of gaps (large arrow) and the disconnection of the liver parenchymal cells (small arrow) becomes apparent. Scale bar, 40 micrometer. The inset illustrates the severely damaged endothelial lining (arrow, gaps; scale bar 1 micrometer).

(i.e., Kupffer cells) in the sinusoids of kava-treated livers (Figure 5, high magnification TEM images). Close examination by TEM revealed that Kupffer cells appear to be swollen and the presence of large cytoplasmic vacuoles and phagocytosed material is apparent. Furthermore, the cells have lost their typical ruffled cell membrane aspect and concurrently they appear to disengage from the sinusoidal endothelial lining.

DISCUSSION

This study provides LM, TEM and SEM evidence that perfusion of rat liver with kavain (10 μg/mL or 43.5 μmol/L) for 2 h causes severe vascular and endothelial damage manifesting as vasoconstriction, gaps and loss of endothelial and subendothelial liver tissue integrity when compared to control livers. The liver parenchymal tissue looks quite normal except for the cells lying close to the Space of Disse (indicating vacuolization). No other differences were observed between the kavain treated and control livers.

This current study does not allow elucidation of the exact mechanisms by which kavain induces the severe ultrastructural changes in the liver. The severe morphological changes were mainly confined to the sinusoidal vascular bed and the parenchymal liver cells lying close to the Space of Disse. This indicates that kavain either affects the endothelial lining directly resulting in the loss of the integrity of the local sinusoidal liver architecture; or alternatively, kavain might activate the local liver-associated macrophage population (i.e., the Kupffer cells) that in turn release cytotoxic substances which cause the acute endothelial vascular- and parenchymal tissue damage. Consistent with this, it is generally accepted that the increased release of pro-inflammatory mediators and reactive oxygen species by stimulated liver macrophages causes severe and acute liver damage[23,24]. In line with this, we collected fine structural evidence showing the presence of activated liver macrophages in livers exposed to kavain. In addition, these data also support the earlier literature observations of the adverse effects of kava on liver function in general[9-13]. Hence these results open up a new avenue for future research to investigate the cellular adverse effects of kava constituents on isolated liver parenchymal-, endothelial-, and Kupffer cells in mono- or co-cultures by using cytotoxic and functional metabolic assays. Furthermore, additional *in vivo* experiments might address the intriguing question of whether the effects of kavain on liver tissue are reversible, and may in part explain why patients recover from kava intoxication. Mounting literature evidence is available that dysfunction of the hepatic sinusoid can be reversed by modulating the Kupffer cell population[25] or its activation status[26]. On the

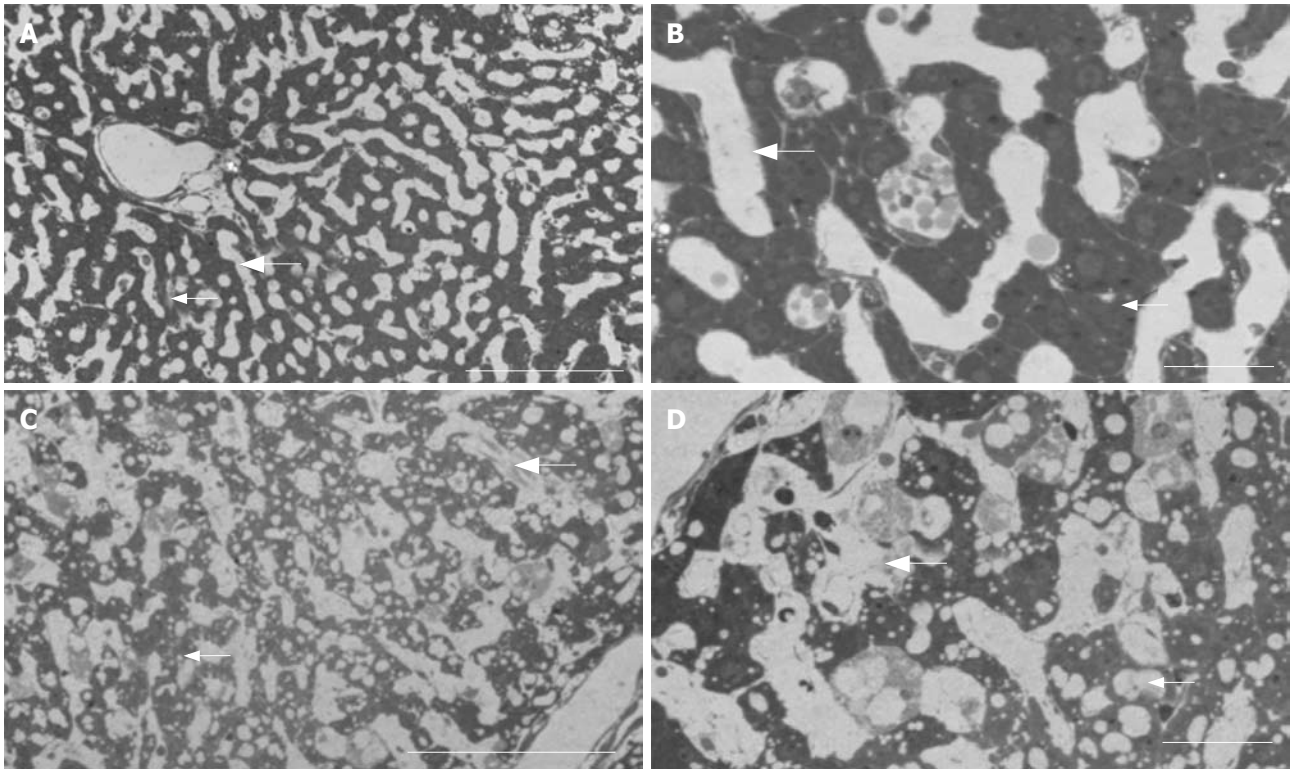


Figure 3 Bright field light optical micrographs of control (**A** and **B**) and kavain-treated livers (**C** and **D**). Note, comparison with the high-magnification TEM data for comparative purposes. **A**: Control liver tissue at low magnification reveals normal histology: i.e., hepatocytes (small arrow) are organized in long cords following the liver sinusoids or microvascular bed (large arrow). Scale bar, 100 micrometer; **B**: Higher magnification of control tissue reveals the patent empty sinusoidal lumen (large arrow) and the relationship with the neighbouring hepatocytes (small arrow). Scale bar 40 micrometer; **C**: In contrast, kavain-treated samples reveal narrowing of the microvessels (large arrow), indicating sinusoidal blood vessel contraction (compare with A). Small arrow, hepatocytes. Scale bar, 100 micrometer; **D**: Intermediate magnification of kavain-treated samples reveal strong vacuolization of the liver parenchymal tissue (small arrow) and loss of normal sinusoidal vasculature (large arrow). Scale bar, 40 micrometer.

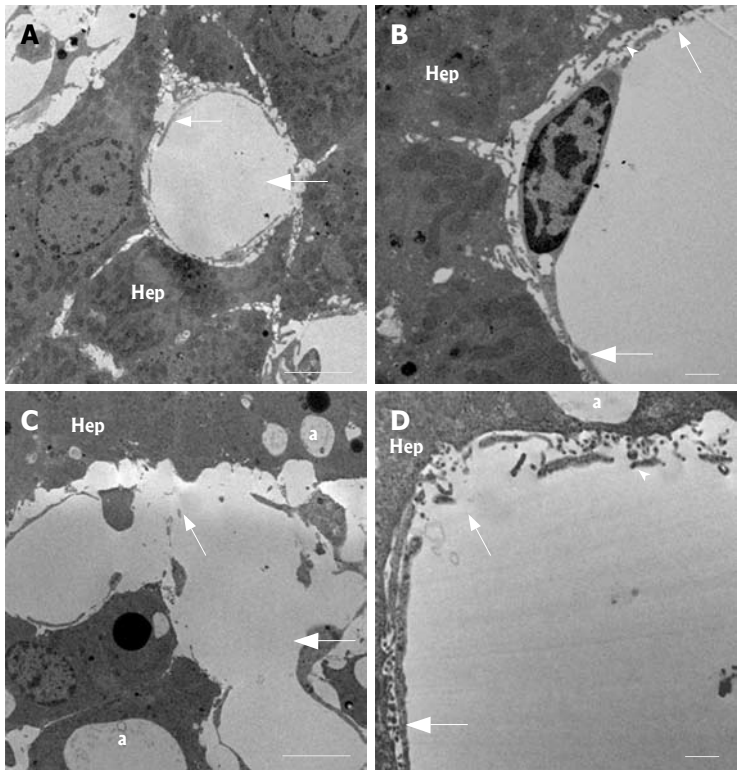


Figure 4 Transmission electron micrographs of control (**A** and **B**) and kavain-treated livers (**C** and **D**). Note, comparisons of these high-resolution TEM data with the corresponding low-magnification light microscopy images. **A**: Intermediate magnification reveals the intact histological relationship between the liver sinusoidal endothelium (small arrow) and the neighbouring liver parenchymal cells (Hep) of control liver tissue. Note the lumen (large arrow) of the small liver capillaries, also called the liver sinusoids. Scale bar, 5 micrometer; **B**: Examination of control samples at higher magnification reveals the intact endothelial lining (large arrow) containing the typical small transportation pores (fenestrae) (small arrow). Hep, hepatocytes; arrowhead, the space of Disse (defined as the space between the endothelial processes and the hepatocytes). Scale bar, 1 micrometer; **C**: Kavain-treated liver tissue samples at intermediate magnification shows strong signs of vacuolization of the hepatic tissue (a), sinusoidal endothelial damage (small arrow). Large arrow, liver sinusoids; Hep, hepatocytes or liver parenchymal cells. Scale bar, 5 micrometer; **D**: High magnification of liver tissue of kavain-treated rats confirm the earlier SEM observations with respect to large gap formation of the endothelial lining (small arrow). Large arrow, sinusoids; arrowhead, space of Disse; asterisk, vacuolization. Scale bar, 1 micrometer.

other hand, it has been recently shown that acute structural and pathophysiological damage of the liver sinusoidal endothelial lining can revert to normal within days[27].

The current study only examined the effects of one kavalactone, kavain on the liver. Hence it is not possible to conclude whether the other five (dihydrokavain,

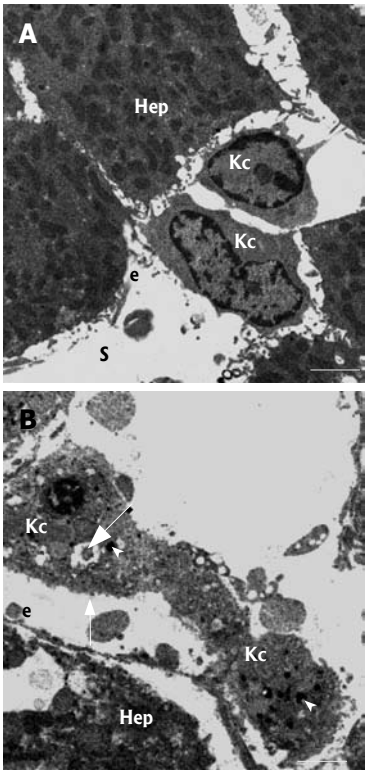


Figure 5 High-magnification transmission electron micrographs of Kupffer cells (Kc) in control (**A**) and kavain-treated livers (**B**). **A**: Kupffer cells are located within the sinusoid (S) and adhere to the endothelial (e) lining by means of multiple cytoplasmic extensions. Note, hepatocytes (Hep); **B**: In kavain-treated livers, Kupffer cells (Kc) show an entire different ultrastructure when compared to control livers (see figure 4A for the difference): i.e., the cells look swollen, contain large amounts of electron dense phagocytosed material (arrowhead), have large vacuoles (large arrow), and have lost their ruffled and microvillous cell surface characteristics (small arrow). Scale bars, 5 micrometer.

methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin) major kavalactones have similar effects individually on the liver. In addition, since kava contains all the six kavalactones in combination, this pilot study has not examined any interactive or synergistic effects of these kavalactones with respect to adverse effects on the liver. It is also not able to be predicted whether kavain and other kavalactones have similar effects on human liver as has been observed here with rat liver.

This study also used only one (10 μg/mL or 43.5 μmol/L) concentration of kavain which was continuously exposed to the livers for 2 h. The choice of this exposure concentration and time was based on literature data. Plasma concentration of total kavalactones was about 33 μg/mL in a fatal human accident subsequent to kava ingestion with alcohol and cannabis[28]. The toxic concentration of kava extracts was 50 μg/mL in rat hepatocytes[29] while kavain toxic concentration (as judged by release of lactate dehydrogenase, LDH) was 125 μg/mL in mouse hepatocytes[30]. Based on this literature data, the kavain concentration in the current study was 10 μg/mL, and the results clearly show liver damage at this concentration. It would therefore be expected that at higher kavain concentrations adverse hepatic effects would be more severe. The exposure time of 2 h reflects literature evidence that livers remain normal during perfusions up to 3 h[18]. The structural changes observed after kavain exposure are therefore genuine as control perfusions revealed normal liver architecture. However, it is not known if the effects of kavain noted here are acute or chronic or if the effects are reversible since the livers were continuously exposed to kavain for 2 h. Since only one kavain concentration was used, it is not known if the effects observed here are concentration-dependent and what the minimum hepatotoxic concentration of kavain is.

ACKNOWLEDGMENTS

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COMMENTS

Background

Since the use of kava and kava-containing products were banned in some Western countries due to case report of liver failure, this study was aimed to examine the vascular, microvascular and hepatocellular fine morphological changes that might occur after perfusion of rat livers with major kavalactone kavain.

Research frontiers

This article demonstrates for the first time using light and electron microscopy liver vascular, microvascular architecture and hepatocellular morphologic changes after liver perfusion with kavain perfusate solution.

Innovation and breakthroughs

The isolation perfused rat liver (IPRL) method has been widely used in drug metabolism studies as it closely simulates *in vivo* (whole body) conditions with the advantage that experimental conditions can be precisely controlled, like perfusion time and drug concentration, since high concentrations of drugs would normally be a limitation because of their side effects *in vivo*. Thus IPRL combined with light and electron microscopic techniques increases the chance of determining the exact ultrastructural changes in the liver.

Applications

In this study kavain solution (10 μg/mL) was perfused for 2 h in IPRL, after standard fixation and tissue preparation, the samples were examined by scanning electron microscopy, transmission electron microscopy and light microscopy. Examinations indicated kavain-treated rat livers displayed severe vascular and endothelial damage compared to control livers. The data so far support the hypothesis that kavain induces adverse effects on liver. Further investigations with other major kavalactones and with different kavalactone concentrations to examine their effects on liver are urgently needed.

Terminology

Herb kava-kava (*Piper methysticum* Forst) has been used extensively in the Pacific as a drink for thousands of years. The roots of kava are used to prepare a drink for social and ceremonial occasions, similar to coffee or tea in other cultures. The root is also used for soothing the nerves, inducing relaxation and sleep. Various preparations of kava have also been marketed since 1980s in Europe and North America to manage mild anxiety and insomnia.

Peer review

The authors show that kavain induces adverse effects. The methods and histological examinations are rigid and instructive. The presentation is good. This present study is an interesting trial.

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BASIC RESEARCH

CO liberated from CORM-2 modulates the inflammatory response in the liver of thermally injured mice

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Abstract

AIM: To explore the effects of CO-releasing molecules [tricarbonyldichlororuthenium (Ⅱ) dimer, CORM-2]-liberated CO on attenuation of inflammatory responses in liver of an experimental animal model of thermal injury and to investigate the associated potential mechanisms.

METHODS: Thirty-six mice were assigned to three groups in three respective experiments. In each experiment, mice in sham group (n = 4) received sham thermal injury, whereas mice in burn group (n = 4) received a 15% of total body surface area (TBSA) full-thickness thermal injury, and mice in burn + CORM-2 group (n = 4) received the same thermal injury with immediate administration of CORM-2 (8 mg/kg, iv). Hepatic tissue sections were stained with hematoxylin and eosin and examined under a light microscope. Levels of aminotransferases (ALT and AST) and nitric oxide (NO) were measured by biochemical methods. Tumor necrosis factor-α (TNF-α) and interleukin (IL-1β) activity, and the protein expression of iNOS and HO-1 in serum and tissue homogenates were assessed. In *in vitro* experiments, Kupffer cells were stimulated with LPS (10 μg/mL) for 4 h in the presence or absence of CORM-2 (10-100 μmol/L). Subsequently, the expression levels of TNF-α and NO production were assessed.

RESULTS: Pro-inflammatory mediators (TNF-α, IL-1β, NO) in serum and liver homogenates of thermally injured mice were significantly reduced by CORM-2 administration. This was accompanied by a decrease in the expression of iNOS while an increase in the expression of HO-1 in the liver tissue. In parallel, the concentrations of TNF-α and NO in supernatants of LPS-stimulated Kupffer cells co-incubated with CORM-2 (10-100 μmol/L) were also markedly decreased. Histological examination demonstrated that CORM-2 could attenuate the leukocytes infiltration to the liver tissue.

CONCLUSION: CORM-released CO modulates liver inflammation and significantly protects liver injury in burn mice by inhibiting the expression of iNOS and NO production, down-regulating the expression of pro-inflammatory mediators (TNF-α, IL-1β).

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Key words: Carbon monoxide; Thermal injury; Liver; Inflammation

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INTRODUCTION

It is well known that endogenous carbon monoxide (CO), a bi-product of inducible heme oxygenase (HO-1), can modulates inflammation. In addition, some experiments have determined that the administration of exogenous CO inhibits lipopolysaccharide (LPS)-induced production of cytokines both *in vivo* and *in vitro*, and consequently exhibits important cytoprotective function and anti-inflammatory properties that are beneficial for the resolution of acute inflammation[1-3].

Recently, transitional metal carbonyls have been identified as potential CO-releasing molecules (CO-RMs) with the potential to facilitate the pharmaceutical use of CO by delivering it to tissues and organs[4]. CO-RMs have been shown to act pharmacologically in rat aortic and cardiac tissue where liberation of CO produced vasorelaxant effects[5-8] and decreased myocardial ischemia-reperfusion damage[9,10], respectively.

Our recent study has confirmed that tricarbonyldichlor oruthenium (Ⅱ) dimer (CORM-2), one of the novel group

of CORMs, inhibited the leukocytes sequestration in liver[11] and down-regulated inflammatory response in the lung[12] of burn-challenged mice. However, the regulating mechanisms of CORM-2 in the inflammatory response in the liver of burn-challenged mice remain unknown. Severe burn injury is a complicated process that involves several host responses, including inflammation, immune response and even systemic inflammatory response syndrome (SIRS). Different cytokines are likely to be produced during these host responses. Some of these cytokines may be produced in response to the thermal injury. Also, the pathophysiological changes seen in SIRS are often not due to the infectious organism itself but instead to the uncontrolled production of pro-inflammatory cytokines and chemokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1b. Accumulation of these mediators leads to leukocyte recruitment, capillary leak and tissue damage, ultimately producing the lethality of sepsis[13-15].

Based on these preliminary observations, in this study, we employed CORM-2 to determine whether it can exert the potential influence to modulate the important inflammatory mediators (TNF-α, IL-1β and NO) in the liver of thermally injured mice.

MATERIALS AND METHODS

Materials

Tricarbonyldichlororuthenium (Ⅱ) dimer (CORM-2) was obtained from Sigma Aldrich and solubilized in dimethyl sulfoxide (DMSO) to obtain a 10 mmol/L stock. Polyclonal or monoclonal antibodies against HO-1 and iNOS were purchased from Santa Cruz Biotechnology Inc. All other chemicals were of analytical reagent grade and obtained from Sigma-Aldrich (Shanghai Trading Co. Ltd.) unless otherwise stated.

Animal and burn protocol

Thirty-six male C57BL/6 mice, weighing 20 ± 2 g, were fed a standard laboratory diet and water *ad libitum*. Mice were assigned to three groups in three respective experiments. In each experiment, mice in sham group (n = 4) received sham thermal injury, whereas mice in burn group (n = 4) received a 15% of total body surface area (TBSA) full-thickness thermal injury and CORM-2 group (n = 4) received the same thermal injury with immediate administration of CORM-2 (8 mg/kg, iv). The experimental protocol was approved by the Council on Animal Care at Jiangsu University on the protection and the welfare of animals. Under anesthesia with spontaneous inhalation of isoflurane-N_2O (Abbott Laboratories, Missisauga, ON, Canada) in a 60% oxygen-40% nitrogen mixture, the dorsum of each mouse was shaved and the mice were subjected to a 15% of total body surface area (TBSA) full-thickness thermal injury as previously described[16,17]. Sham animals were immersed in a room temperature water bath. All animals were resuscitated with 1.5 mL saline immediately after thermal (or sham) injury. No wound care was required for the burn wounds. This burn method achieves a histologically proven, full-thickness scald injury[18,19]. The animals were sacrificed at 24 h after experimental manipulation.

Histologic studies

The liver specimens harvested from different groups of mice were immersed in 40 mL/L formaldehyde solution after 24 h of the thermal injury. The tissues were embedded in paraffin wax, serially sectioned, and stained with hematoxylin-eosin. Liver morphologic characteristics were evaluated under light microscope.

Isolation and culture of Kupffer cells

In *in vitro* experiment, the effect of CORM-2 on Kupffer cells was studied by isolating Kupffer cells from the normal livers. Liver perfusion was performed using a modification of the two-step collagenase perfusion technique introduced by previous studies[20,21]. Briefly, the liver was perfused through the portal vein with D-Hank's solution until became blood-free, and then with Hank's solution containing 0.5 g/L collagenase Ⅳ. The latter was administered by recirculation until the vessels were digested (up to 20 min). The liver was then scraped using a cell scraper, filtered through a 100-μm filter, and stirred in Hank's solution containing 2.5 g/L pronase and 0.05 g/L Dnase at 37℃ for 20 min. After three times of centrifugation and washing at 300 r/min for 10 min at 4℃ in Gey's balanced salt solution (GBSS), cells were centrifuged in an 180 g/L Nycodenz gradient at 2500 r/min for 20 min. Kupffer cells were carefully sucked by cusp-straws at the pearl layer inderphase. Purified Kupffer cell fractions were then obtained by centrifugal elutriation. The viability of Kupffer cells prepared was more than 95% as determined by trypan blue exclusion. The purity of Kupffer cells was greater than 90% based on a peroxidase activity assay[22].

Kupffer cells were cultured in RPMI 1640 medium containing antibiotics (penicillin 100 U/mL; streptomycin 100 mg/mL), 2 mmol/L glutamine, and 100 mL/L fetal calf serum. The cells at a density of 1×10^6/mL were cultured in 24-well culture plates and co-incubated with LPS (10 μg/mL) and different concentrations of CORM-2 (10, 50, 100 μmol/L) for 4 h. The supernatants were collected and TNF-α and NO concentrations were measured using the methods described below.

Measurement of serum level of transaminases, TNF-α and IL-1β levels in serum, tissue homogenate and cell culture supernatants

Blood samples were obtained by cardiac puncture of the left ventricle. The samples were stored in serum tubes (Capiject, Terumo Medical Corporation, USA) and immediately centrifuged at 6500 r/min for 5 min. Evaluation of hepatocellular injury was performed by determining the enzymatic activity of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum samples using a commercial kit (Boehringer Manheim, Munich, Germany) according to the manufacturer's instructions. Concentrations of TNF-α and IL-1β levels in serum, tissue homogenate and supernatants of cell culture were assayed by enzyme-linked immunosorbent assay kits (Genzyme, Corp., Cambridge, MA) following the manufacturer's instructions.

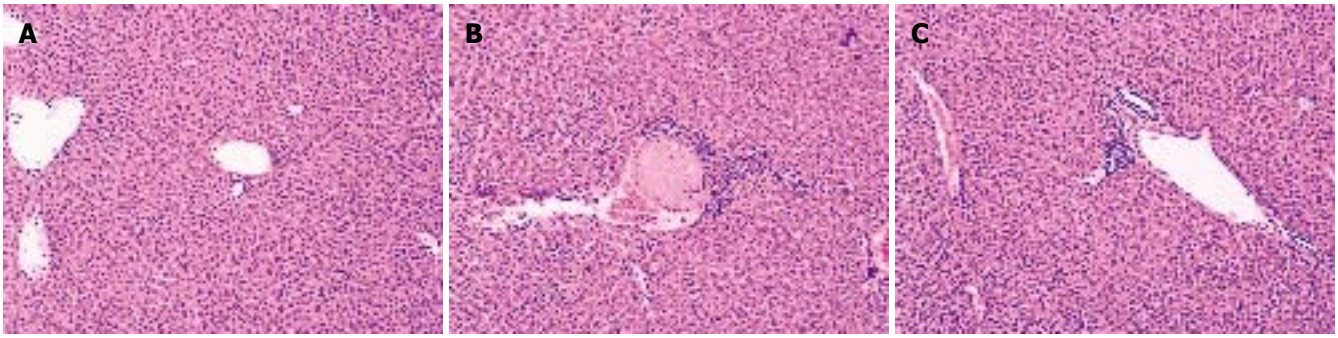


Figure 1 Histologic results of liver tissue after staining with hematoxylin and eosin (HE, × 100). Mice were challenged with thermal injury and immediately intravenously injected with CORM-2 (8 mg/kg). **A**: Sham group; **B**: Burn group; **C**: Burn + CORM-2 group.

Measurement of NO in serum, tissue homogenate and cell culture supernatants

Nitric oxide (NO) was measured by a microplate assay using Griess reagent, which produces a chromophore with the nitrite[23]. Briefly, 100 μL of supernatant was removed and incubated with 100 μL of Griess reagent (10 g/L sulfanilamide and 1 μg/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 (A_{540}) was measured using a microplate reader (Bio-Tek, USA). 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. Results were expressed as μmol/L.

Western blot analysis

Tissues were homogenized for extract preparations in ice-cold mild lysis buffer containing 10 mL/L Nonidet P-40, 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate (pH 7.2), 2 mmol/L EDTA, 50 mmol/L sodium fluoride, 0.2 mmol/L sodium vanadate, and 1 μg/mL aprotinin. The tissue homogenates were centrifuged at 20000 × *g* for 15 min and supernatants were collected. SDS-PAGE was performed on equivalent amounts of protein samples using precast 7% resolving/4% stacking Tris-HCl gels (Bio-Rad, Hercules, CA). Separated proteins were then transferred onto PVDF membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Membranes were blocked in 50 g/L nonfat milk in TBST (TBS buffer containing 1 g/L Tween 20) for 1 h at room temperature. Blocked membranes were incubated in primary antibodies specific for mouse iNOS (1:2000 dilution) and HO-1 (1:5000 dilution) in TBST overnight at 4℃. Then the membranes were washed and probed with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Inc.) 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. The specific bands were quantified by densitometry (Bio-Rad GS-710 Densitometer).

Table 1 Liver injury estimated from serum ALT and AST levels

Groups	*n*	AST (IU/L)	ALT (IU/L)
Sham	6	70.3 ± 6.8	38.5 ± 6.1
Burn	6	345.6 ± 32.7[b]	107.7 ± 16.9[b]
Burn + CORM-2	6	189.9 ± 24.3[a]	58.6 ± 9.7[a]

[b]$P < 0.01$ *vs* sham; [a]$P < 0.05$ *vs* burn.

Statistical analysis

Data were expressed as mean ± SE. Statistical analysis was performed using ANOVA and Student's *t*-test for the comparisons. A P value less than 0.05 was considered statistically significant.

RESULTS

Histology

Histological analysis showed that the liver sections from sham mice demonstrated a normal architecture, while thermal injury induced marked vacuolization and infiltration of the inflammatory cells to the tissue (Figure 1). After *in vivo* administration of CORM-2 (8 mg/kg, iv), granulocyte infiltrations were significantly decreased. However, CORM-2 did not improve the thermal injury-induced congestion in liver sinusoids.

Effect of CORM-2 on transaminases levels in burn-challenged mice serum

Hepatocyte injury was evaluated by determining the serum concentrations of ALT and AST. At 24 h after a 15% TBSA full-thickness thermal injury, serum hepatic transaminases (ALT and AST) were found to be markedly increased in the burn mice as compared to the sham mice ($P < 0.05$), whereas the corresponding values in the burn mice receiving CORM-2 (8 mg/kg, iv) at the beginning of resuscitation were significantly decreased as compared to the burn mice without CORM-2 administration ($P < 0.05$, Table 1).

Effect of CORM-2 on TNF-α and IL-1β levels in serum and tissue homogenates of burn-challenged mice

At 24 h after a 15% TBSA full-thickness thermal injury,

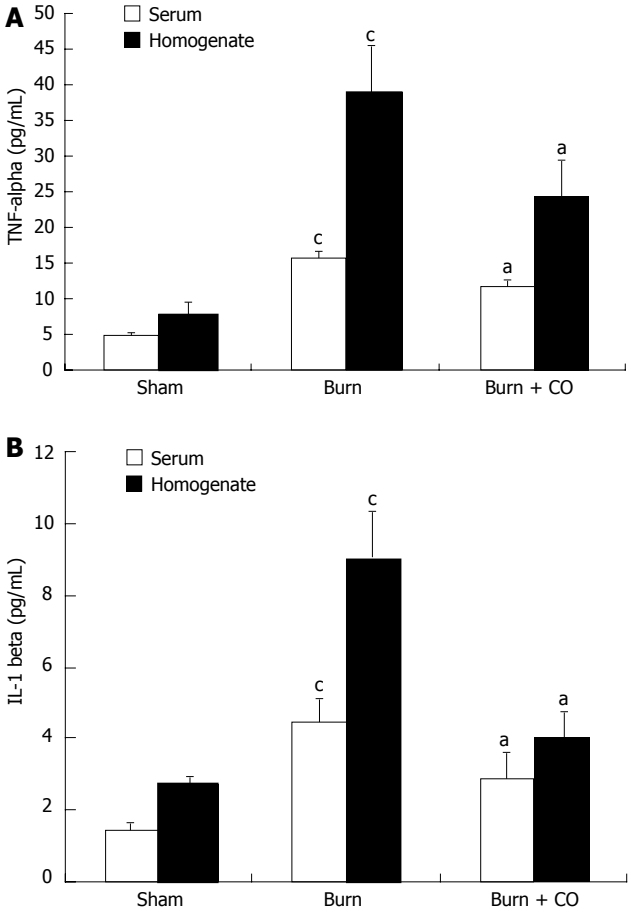


Figure 2 Effect of CORM-2 on TNF-a and IL-1β levels in serum and tissue homogenates of burn-challenged mice. Data are expressed as mean ± SE of three experiments (three mice per group). $^{a}P < 0.05$ *vs* burn group; $^{c}P < 0.05$ *vs* sham group.

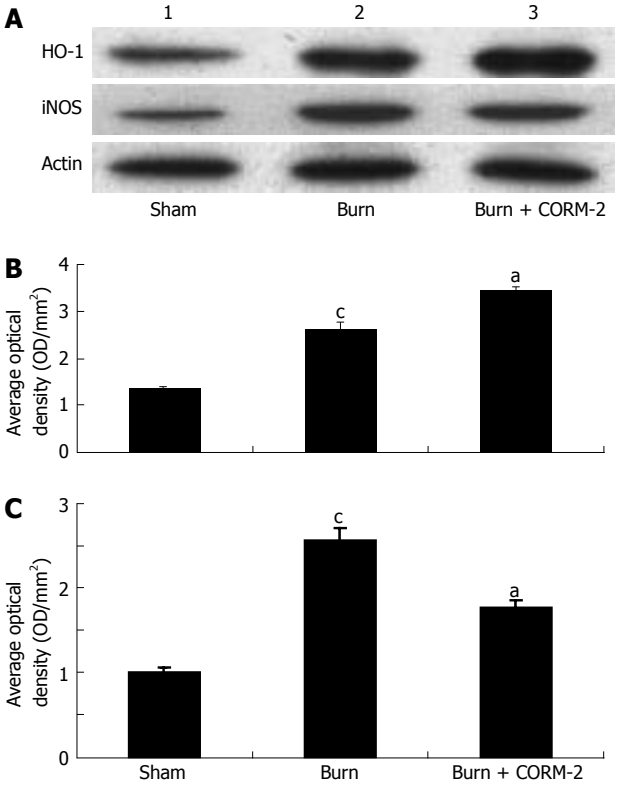


Figure 3 Effects of CORM-2 on protein expression of iNOS and HO-1 in the liver of burn-challenged mice. Protein expressions of iNOS and HO-1 were performed by Western blotting at 24 h after thermal injury. **A**: Representative experiment; **B** and **C**: Quantitative results (average absorbance, A_{540}) of three. $^{a}P < 0.05$ *vs* burn group; $^{c}P < 0.05$ *vs* sham group.

TNF-α and IL-1β levels in serum and tissue homogenates of the burn-challenged mice were markedly increased compared to the sham mice. After administration of CORM-2, the elevation in serum and tissue homogenates levels of TNF-α and IL-1β was significantly abolished (Figure 2A and B).

Effect of CORM-2 on expression of iNOS and HO-1 in the liver of burn-challenged mice

At 24 h after a 15% TBSA full-thickness thermal injury, the expression of iNOS in liver tissue significantly increased compared to the sham mice. *In vivo* administration of CORM-2 (8 mg/kg, iv) resulted in a significant decrease in iNOS expression (Figure 3A and C). HO-1 expression in the liver of burn mice was markedly increased compared to sham mice. Interestingly, HO-1 expression in the liver of burn mice with administration of CORM-2 was more significantly increased as compared to the burn group (Figure 3A and B).

Effect of CORM-2 on NO production in serum and tissue homogenates of burn-challenged mice

As shown in Figure 4, production of NO was low in sham group. After burn challenge, NO levels in serum and tissue homogenates were significantly increased ($P < 0.05$ *vs* sham), whereas administration of CORM-2 significantly decreased the NO levels ($P < 0.05$ *vs* burn group).

Effect of CORM-2 on NO and TNF-α levels in supernatants of Kupffer cells stimulated with LPS

As shown in Figure 5, NO and TNF-α levels were low in controls. After Kupffer cells were stimulated by LPS (10 μg/mL) for 4 h, NO and TNF-α levels in supernatants were significantly increased as compared to controls ($P < 0.05$), while markedly decreased when Kupffer cells were co-incubated with LPS and CORM-2 ($P < 0.05$ *vs* LPS group).

DISCUSSION

Recent studies have demonstrated various physiologic and immunologic alterations in acute inflammations associated with infection, trauma and thermal injury[24-27]. Increasing attention has been directed to the role of cytokines in the mechanisms of such alterations. TNF-α, IL-1β, IL-6, which are pro-inflammatory cytokines, are considered potential mediators of inflammation produced by immunoregulatory cells as well as by a variety of other cell types[28], and play a major role in the regulation of immune response and inflammation[29].

It is well known that heme oxygenase-1 (HO-1), the inducible isoform of heme oxygenase which catalyzes the formation of the endogenous carbon monoxide (CO),

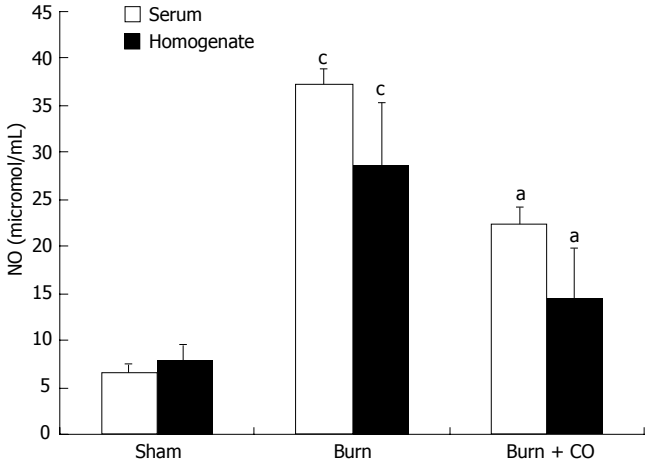


Figure 4 Effects of CORM-2 on NO level in serum and tissue homogenates of burn-challenged mice. Measurement of NO level was performed by a microplate assay using Griess reagent at 24 h after thermal injury. NO levels both in serum and tissue homogenates of thermally injured mice were markedly increased, but significantly inhibited by *in vivo* administration of CORM-2. $^{a}P < 0.05$ *vs* burn group; $^{c}P < 0.05$ *vs* sham group.

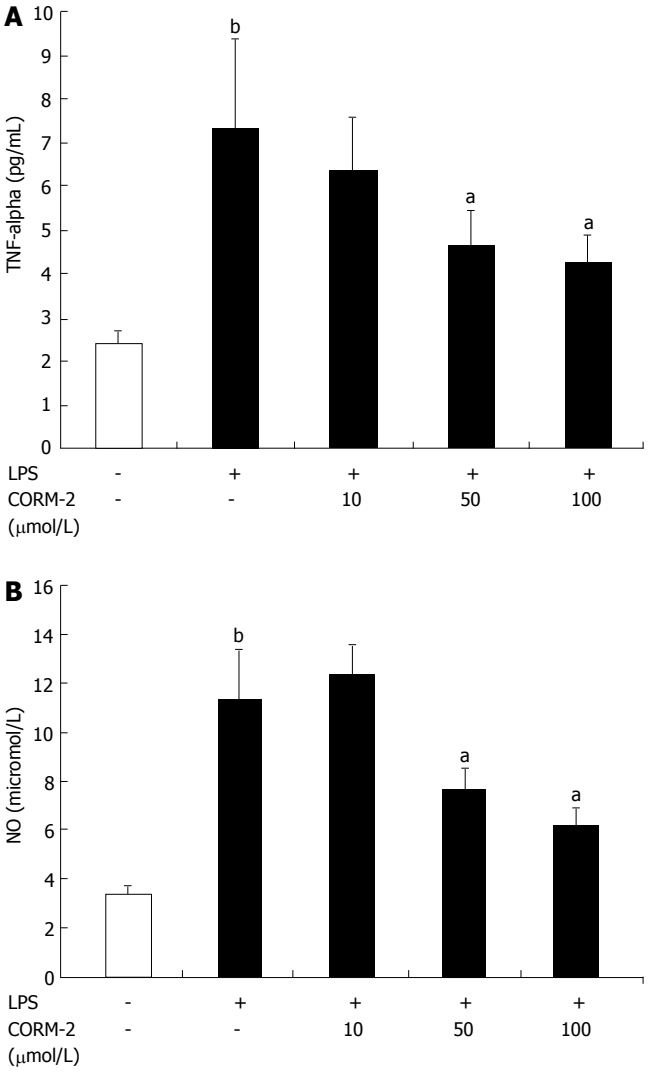


Figure 5 Effect of CORM-2 on levels of NO and TNF-α in supernatants of Kupffer cells stimulated with LPS. Kupffer cells were co-incubated with LPS and different concentrations of CORM-2 (10-100 mmol/L) for 4 h. All values are expressed as means ± SE ($n = 3$). $^{b}P < 0.01$ *vs* control; $^{a}P < 0.05$ *vs* LPS group.

biliverdin/bilirubin and ferrous iron, exhibits important anti-inflammatory properties that are beneficial for the resolution of acute inflammation[30]. Many experimental studies have highlighted the specific and independent role of exogenous CO (i.e. CO gas inhalation) in the modulation of inflammation[31]. Recently some new metal carbonyl-based compounds (CO-releasing molecules, CO-RMs) that have the ability to release CO in biological systems have been identified and synthesized. The vasoactive, antihypertensive and antirejection effects of CO-RMs have been demonstrated to be due to the CO liberated by the compounds. CORM-2, a DMSO-soluble CO-RM, also has exhibited anti-inflammatory actions in an *in vitro* model of LPS-stimulated murine macrophages. Thus, the aim of this study was to shed light on the role of CORM-2, as a exogenous CO, on the local and systemic inflammatory response induced by burn injury.

In this study, we found that HO-1 was significantly up-regulated by thermal injury. Interestingly, HO-1 expression in the liver of thermal injury with administration of CORM-2 was more significantly increased compared to burn mice (Figure 3). This result indicated that not only major burn injury, a severe trauma, might significantly induce HO-1 expression, but also the increase of HO-1 expression can be further enhanced by the *in vivo* administration of CORM-2. Through the by-product (CO, and/or biliverdin), the potent cytoprotective and anti-inflammatory functions were ultimately led to exert.

The data from this study showed that thermal trauma induced hepatocellular injury as evidenced by increased plasma ALT and AST levels. The *in vivo* administration of CORM-2 attenuated post-burn hepatocellular injury (Table 1). In parallel, after application of CORM-2, the elevation both in serum and tissue homogenates levels of TNF-α and IL-1β in burn-challenged mice was abolished (Figure 2). This result suggests that CORM-2 in the prevention of burn-induced liver damage may involve in down-regulation of the production of TNF-α, since TNF-α is an important mediator of hepatic inflammation in burn-induced liver damage. An alternative possibility is that the anti-inflammatory effects of CORM-2 are owing to its potential inhibition of the production and secretion of TNF-α. These data indicated that CORM-2 exhibits, at least partly, an important cytoprotective function during the early period of thermal injury.

Based on current research findings, oxidative stress is believed to be the major causative agent to damage the organs distant from the original burn wound. Nitrosative stress initiates an inflammatory cascade that includes acute phase protein synthesis, up-regulation of inflammatory adhesion molecules, and pro-inflammatory cytokine release[32,33]. Thus, tissue or organ injury after thermal trauma appears to be mediated both by reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as hydroxyl radical, superoxide anion, hydrogen peroxide, and peroxynitrite. It has been demonstrated that burn injury is associated with lipid peroxidation mediated by ROS and is believed to be an important cause of oxidative damage to cellular membranes and, eventually, cell death[34]. Also,

nitric oxide (NO) plays a crucial role in cellular injury in burns. It has been recognized that NO is produced by cNOS and/or iNOS in mice liver[35,36]. In this study, we found that thermal injury produced a significant up-regulation in the inducible nitric oxide synthetase (iNOS) expression (Figure 3), thereby up-regulating iNOS-mediated nitric oxide production. After administration of CORM-2, the iNOS protein expression was significantly decreased, it might effectively decrease the overabundance of NO, which produces significant cellular toxicity *via* several mechanisms[37].

Lipopolysaccharide (LPS), a constituent of the Gram-negative bacterial cell wall, is the leading cause of sepsis, and when administered experimentally to macrophages or mice, mimics the same inflammatory responses. After LPS administration, there is a rapid increase in the pro-inflammatory mediators. The present study showed that the levels of TNF-α and NO in the supernatants of Kupffer cells stimulated by LPS were markedly increased compared to controls, while this increases was down-regulated after the co-incubation with CORM-2 in a concentration-dependent manner (Figure 5). These results are in accordance with our *in vivo* experimental data, which indicated that CORM-2 effectively inhibited TNF-α expression and NO production both in serum and tissue homogenates of experimental models.

In summary, the present study clarifies the anti-inflammatory and cytoprotective role of CORM-2, one of the novel CO-releasing molecules. Application of CORM-2 on thermally injured mice inhibited the expressions of TNF-α and IL-1β both in serum and tissue homogenates of experimental models. Also, iNOS protein expression in liver tissue was effectively inhibited in burn-challenged mice with treatment of CORM-2, this was accompanied by a decrease in NO level. In parallel, TNF-α and NO levels were decreased in LPS-stimulated Kupffer cells treated with CORM-2. Taken together, these findings indicate that CORM-released CO modulates liver inflammation in burn-challenged mice by interfering with TNF-α, IL-1β and NO production, and iNOS and HO-1 protein expressions, thereby suppressing inflammatory response in the liver of experimental models. Further studies are required to understand the detail mechanisms of anti-inflammatory effects mediated by CORMs, and to contribute to the development of a therapeutic approach to protect the liver during severe burn injury.

COMMENTS

Background

Recent studies have shown various physiologic and immunologic alterations in acute inflammations associated with infection, trauma and thermal injury. Increasing attention has been directed to the role of cytokines in the mechanisms of such alterations. TNF-α, IL-1β, IL-6, which are pro-inflammatory cytokines, are considered potential mediators of inflammation produced by immunoregulatory cells as well as by a variety of other cell types, and play a major role in the regulation of immune response and inflammation. In our laboratory, it was confirmed that tricarbonyldichlororuthenium (Ⅱ) dimer (CORM-2), one of the novel group of CORMs, inhibited the leukocytes sequestration in liver and down-regulated inflammatory response in lung of burn-challenged mice. However, the regulating mechanisms of CORM-2 in the inflammatory response in liver of burn-challenged mice remain unknown. Severe burn injury is a complicated process that involves several host responses, including inflammation, immune response and even SIRS. Different cytokines are likely to be produced during these host responses. Some of these cytokines may be produced in response to the thermal injury. Also, the pathophysiological changes seen in SIRS are often not due to the infectious organism itself but instead to the uncontrolled production of pro-inflammatory cytokines and chemokines, including TNF-α, IL-1β.

Research frontiers

Major burn alters immune function, producing an imbalance between pro- and anti-inflammatory cytokine synthesis and increasing susceptibility to post-burn infection and sepsis. Also, severe burn causes damage to multiple organs distant from the original burn wound, leading to multiorgan failure, a serious clinical problem. Liver is one of the most sensitive tissues to ischemia and reperfusion (I/R) injury induced by thermal injury. Different cytokines may be produced in response to the thermal injury. Also, the pathophysiological changes are often not due to the infectious organism itself but instead to the uncontrolled production of pro-inflammatory cytokines and chemokines, including TNF-α, IL-1β. Accumulation of these mediators leads to leukocyte recruitment, capillary leak and tissue damage, ultimately producing the lethality of sepsis.

Innovations and breakthroughs

This is probably the first study which demonstrated CORM-released CO modulates the inflammatory response in the liver of thermally injured mice and the possible associated mechanisms.

Applications

Our research observed that CORM-released CO modulates liver inflammation and significantly protects liver injury in burn mice by inhibiting the expression of iNOS and NO production, down-regulating expression of pro-inflammatory mediators, including TNF-α, IL-1β.

Terminology

Carbon monoxide-releasing molecules (CO-RMs): Transitional metal carbonyls have been identified as potential CO-releasing molecules with the potential to facilitate the pharmaceutical use of CO by delivering it to tissues and organs.

Peer review

This study seems to be well performed, the methods are adequate. The reason for the study seems sound and solid, the conclusions seem to be relevant and the literature covers the subject.

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BASIC RESEARCH

Discovering differential protein expression caused by CagA-induced ERK pathway activation in AGS cells using the SELDI-ProteinChip platform

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Abstract

AIM: To identify the protein expression differences related to the CagA-induced ERK pathway activation in AGS cells.

METHODS: Human AGS cells transfected with *cagA* and blank vector were treated with specific mitogen-activated protein kinase kinase (MEK) inhibitor. Total cell proteins were combined by strong anion exchange (SAX2) and weak cation exchange (CM10) ProteinChip arrays and analyzed using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) proteomics technology. Protein expression profiles were compared with those of inhibitor-untreated *cagA* transfectants. SwissProt/TrEMBL database searching for differentially expressed proteins was carried out using the TagIdent tool with the pI and mass information.

RESULTS: When a total of 16 proteins that showed expression differences in inhibitor-untreated *cagA* transfectants were compared with vector transfectants, three proteins with m/z 4229, 8162 and 9084 were found to have no expression differences after treatment with MEK inhibitor, while the other 13 maintained the same expression differences after inhibitor treatment. Seven pieces of meaningful matching information for the three proteins were obtained from database searching.

CONCLUSION: Biomarkers with m/z 4229, 8162 and 9084 are ERK1/2 phosphorylation dependent, and therefore are the downstream molecules of ERK1/2 in the ERK/MAPK signaling pathway. The three biomarkers may be important cancer-associated proteins according to SwissProt/TrEMBL database information.

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Key words: CagA; ERK pathway; SELDI-TOF-MS; ProteinChip

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INTRODUCTION

H pylori infection, a primary etiological cause leading to gastritis and peptic ulcer[1], is also a high risk factor for gastric cancer, and is classified as a class I carcinogen by the International Research Agency on Cancer (IRAC)[2-6]. Cytotoxin-associated gene A (CagA) protein, which is encoded by cytotoxin-associated gene pathogenicity island, is the major virulence factor of type I *H pylori*. Epidemiological studies indicate that CagA plays an important role in the development of *H pylori*-induced disease, especially in gastric cancer. The CagA antibody titer in gastric cancer patients is higher than in healthy individuals. Similarly, CagA antibody titer is elevated in high-risk populations[7]. Compared with *cagA*-negative *H pylori* strains, *cagA*-positive strains dramatically increased the occurrence of severe gastritis and gastric adenocarcinoma[8-12]. Experimental studies reveal that CagA is translocated into host cells through a type Ⅳ secretion system of *H pylori*[13]. After being tyrosine-phosphorylated at the C-terminal repeat sequence(s) by c-src/Lyn kinase[14,15], CagA strongly stimulates the mitogen-activated protein kinase (MAPK) signaling pathway in host cells, through interaction with SHP-2, a tyrosine phosphatase containing an SH2 homolog, and

results in cell proliferation and differentiation[16]. As one of the major three members of the MAPKs, extracellular signal-regulated kinase (ERK) has been reported to be a key molecule in cell carcinogenesis in various cell types[17-19]. Higashi *et al*[20] have found that ERK is activated by CagA *via* SHP-2 recruitment and activation in AGS cells (human gastric adenocarcinoma epithelial cell line). Our previous research further proved that transformation of immortalized gastric epithelial cells by CagA takes place through the ERK/MAPK pathway[21]. However, the precise mechanism of the interactions between CagA and host cells in associated pathogenesis has not been fully elucidated.

Recently, gene microarrays have been used to establish the global pattern of gene expression in *cagA* gene-transfected AGS cells[22]. Nevertheless, gene and protein expression levels cannot easily be correlated or equated since proteins can exist in different posttranslational functional states. Therefore, it is important to gain an overall view of host response to CagA interference at the protein level. However, to date, few studies that exploit the global protein-expression pattern that can reflect host-cell response to CagA have been reported. Based on surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) technology, the ProteinChip platform has recently been shown to be valuable in establishing protein expression profiles and discovering new biomarkers[23-25]. ProteinChips, on which proteins from biological samples are selectively retained according to their biochemical properties, are analyzed directly by MS to form peak patterns as protein-expression profiles. The location and intensity of every peak in the pattern reflect the molecular weight and abundance of the corresponding protein. Therefore, the patterns can be used to analyze the protein expression differences under different conditions. This innovative technique has certain advantages over other traditional methods such as two-dimensional gel electrophoresis in its small sample volume, high throughput capability, subfemtomole range sensitivity, high resolution at low mass range, and easy operation, thus it has been successfully applied in new biomarker discovery for early diagnosis of cancer and basic research into disease mechanisms[26-29].

The proteome of AGS cells transfected with *cagA* gene was analyzed using a SELDI-ProteinChip platform in our previous study. Sixteen biomarker proteins involved in CagA induced pathogenesis were identified[30]. In this study, we further investigated the relationship of these protein expression differences and activation of the ERK/MAPK signaling pathway by CagA. Three more pivotal disease-related biomarkers among the 16 were further figured out.

MATERIALS AND METHODS

Expression vectors

Wild-type (WT) *cagA*/pcDNA3.1(+) plasmid (WT-*cagA*) and phosphorylation site mutant *cagA*/pcDNA3.1(+) plasmid (MT-*cagA*) were constructed as previously described[31,32]. Briefly, WT-*cagA* was constructed for encoding CagA proteins containing three tandem Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, and one repeated EPIYA motif sequence with Asp-Phe-Asp (DFD) in the D2 region. MT-*cagA* was generated from WT-*cagA* by substituting a phenylalanine codon for a tyrosine codon in the repeated EPIYA motif.

Cell culture and transfection

Human gastric adenocarcinoma epithelial AGS cells (ATCC, Rockville, MD, USA) were cultured in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (Gibco) in six-well plates. For ERK-associated analysis, cells were transiently transfected with WT-*cagA* or blank vectors. For each well, 4 μg plasmids were transfected into 5.5×10^5 cells by using 6 μL Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. WT-*cagA* transfectants, vector transfectants and untransfected cells were divided into three groups that were respectively treated with the specific mitogen-activated protein kinase kinase (MEK) inhibitor U0126 (Sigma, St. Louis, MO, USA) at 10 μmol/L, DMSO vehicle, or were not treated. Cells were collected after 48 h for protein expression analysis. The transfection efficiency was at least 37%, as determined by the cells transfected with EGFP/pcDNA3.1(+). The expression of *cagA* gene was confirmed by RT-PCR.

Western blot analysis

WT-*cagA* transfectants, vector transfectants and untransfected AGS cells were incubated in 10% FCS for 0, 1 or 3 h following a 24-h serum-starvation period. After washing three times with PBS, cells were harvested and treated with lysis buffer (20 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 10 mmol/L EDTA, 10 mL/L Triton X-100, 40 g/L SDS, and 100 mL/L glycerol) at 100℃ for 5 min and then centrifuged at 15000 × *g* for 5 min. Western blot analysis was performed with standard techniques, using anti-total ERK1/2 antibody (1:1000; Cell Signaling, Beverly, MA, USA), anti-phospho-ERK1/2 antibody (1:1000; Cell Signaling), followed by an anti-mouse secondary antibody (Beijing Zhongshan Biotechnological, China). Samples were separated on 10% sulfate-polyacrylamide gel and electrotransferred onto nitrocellulose membranes (Amersham Biosciences, USA). Immunoreactive bands were visualized with the ECL detection system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Kodak X-ray film.

Preparation of total cell lysates

AGS cells of each group were harvested after PBS washing and resuspended in lysis buffer (8 mol/L urea, 40 g/L CHAPS, 50 mmol/L DTT, 40 mmol/L Tris-HCl) containing protease inhibitor cocktail (Sigma). After 30 min lysis on ice, total cell lysates were obtained by centrifugation at 20000 × *g* for 30 min to remove insoluble debris. Protein concentration was measured using BioRad DC protein assay kit (BioRad, Hercules, CA, USA) and then adjusted to 5 mg/mL for further protein expression analysis.

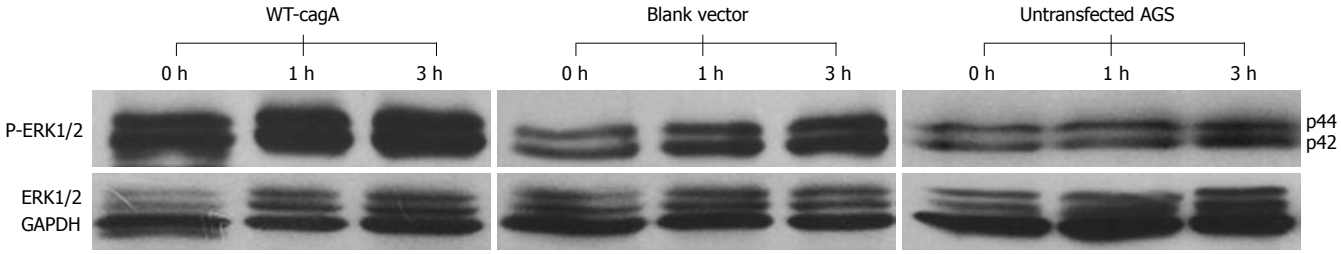


Figure 1 Detection of phosphorylated ERK1/2 in transfected AGS cells. Cells transfected with WT-*cagA* or blank vectors were stimulated with serum by adding fresh medium containing 10% FCS for 0, 1 and 3 h, after 24 h serum starvation, and then harvested for Western blot analysis of total and phosphorylated ERK1/2 (P-ERK1/2). The level of P-ERK1/2 in WT-*cagA* transfectants was significantly higher than that in blank vector transfectants and untransfected AGS cells at all three time points of serum stimulation. No obvious increases in total ERK among the three groups were found. GAPDH level was used as a loading control.

SELDI protein profiling

Expression profiles of each group were obtained by strong anion exchange (SAX2) and weak cation exchange (CM10) ProteinChip Arrays (Ciphergen Biosystems, Fremont, CA, USA) using an "*in situ*" protocol provided by the manual. Before sample loading, each spot of SAX2 and CM10 arrays were equilibrated with 3 μL of binding buffer (50 mmol/L Tris-HCL, pH 9.0 for SAX2 and 50 mmol/L sodium acetate, pH 4.0 for CM10). After 5 min incubation for three consecutive periods, 5 μL total cell lysate (diluted to 1.5 mg/mL in binding buffer) was added for chip binding. All arrays were incubated in a humidity chamber for 1 h and each spot was washed three times with 3 μL binding buffer. After rinsing quickly with HPLC water twice, the arrays were air-dried. One microliter of saturated sinapinic acid (Ciphergen Biosystems) solution in 500 mL/L acetonitrile, in water containing 5 mL/L trifluoroacetic acid, was applied twice, followed by air-drying. Mass accuracy was calibrated using the All-in-1 peptide and All-in-1 protein molecular mass standards (Ciphergen Biosystems). MS analysis of each sample was performed using the ProteinChip Biology System Reader (Model PBS Ⅱ; Ciphergen Biosystems) at a laser intensity of 180 and a detector sensitivity of 7.

Data analysis

Data of every spectrum were collected and analyzed by ProteinChip Software version 3.0 (Ciphergen Biosystems) with integrated Biomarker Wizard software (Ciphergen Biosystems). In brief, baselines were subtracted and normalization was performed by total ion current normalization function, following the software instructions. Biomarker Wizard was then used to identify corresponding peaks in the spectra. Significant differences ($P < 0.05$) in peak intensity of particular proteins between two sample groups were calculated by the software using a Mann-Whitney non-parametrical test.

RESULTS

ERK activity in transfected cells

Western blot results showed that the phosphorylation level of ERK1/2 in WT-*cagA* transfectants was significantly higher than that in vector transfectants and untransfected AGS cells, after serum stimulation at all time points (Figure 1), which indicates that phosphorylated CagA can strongly enhance the phosphorylation level of ERK1/2 in host cells.

Profiling of cell lysates

Protein profiles of cell lysates from each group were obtained from SAX2 (strong anion exchange) and CM10 (weak cation exchange) ProteinChip Arrays. Each type of array surface retains different groups of proteins depending on the surface properties (SAX2 captures proteins with low pIs, while CM10 captures proteins with high pIs), therefore assuring that more host proteins are analyzed. MS analysis was carried out under a laser intensity of 180 and a detector sensitivity of 7.0, which brought out the highest resolution determined by previous experiments. Approximately 200 separate protein peaks with a signal to noise ratio > 5.0 and intensity > 1.0 were obtained in the mass range of 2000-20 000 m/z (mass/charge) in every spectrum, using either CM10 or SAX2 chips.

Biomarker proteins dependent on activation of ERK/MAPK signaling pathway detected by SELDI-TOF-MS

In our previous study[30], we transfected AGS cells with WT-*cagA*, phosphorylation site mutant *cagA*, and blank vector. Protein-expression profiles were analyzed using a SELDI-ProteinChip platform, in order to gain an insight into the effect of CagA and CagA phosphorylation on host protein expression. A total of 16 proteins in AGS cells were found have expression differences under the effect of CagA, and 12 of these were CagA phosphorylation dependent (Figure 2, Table 1). These biomarkers may contribute to the host response to CagA and may be involved in CagA-induced pathogenesis.

According to the previous results, most changes in expression after *cagA* transfection are related to CagA phosphorylation. Phosphorylated CagA can interfere with signal transduction of the ERK/MAPK pathway that plays an important role in CagA-induced cell proliferation and transformation. Various downstream molecules involved in the pathway need to be identified. Thus, our next question is which biomarkers among the 16 are caused by CagA-induced activation of the ERK/MAPK signaling pathway. The results may help us to focus on the most important proteins involved in the host response to CagA. After confirming by Western blotting that WT CagA can significantly increase the activation of ERK1/2, we treated AGS cells with MEK

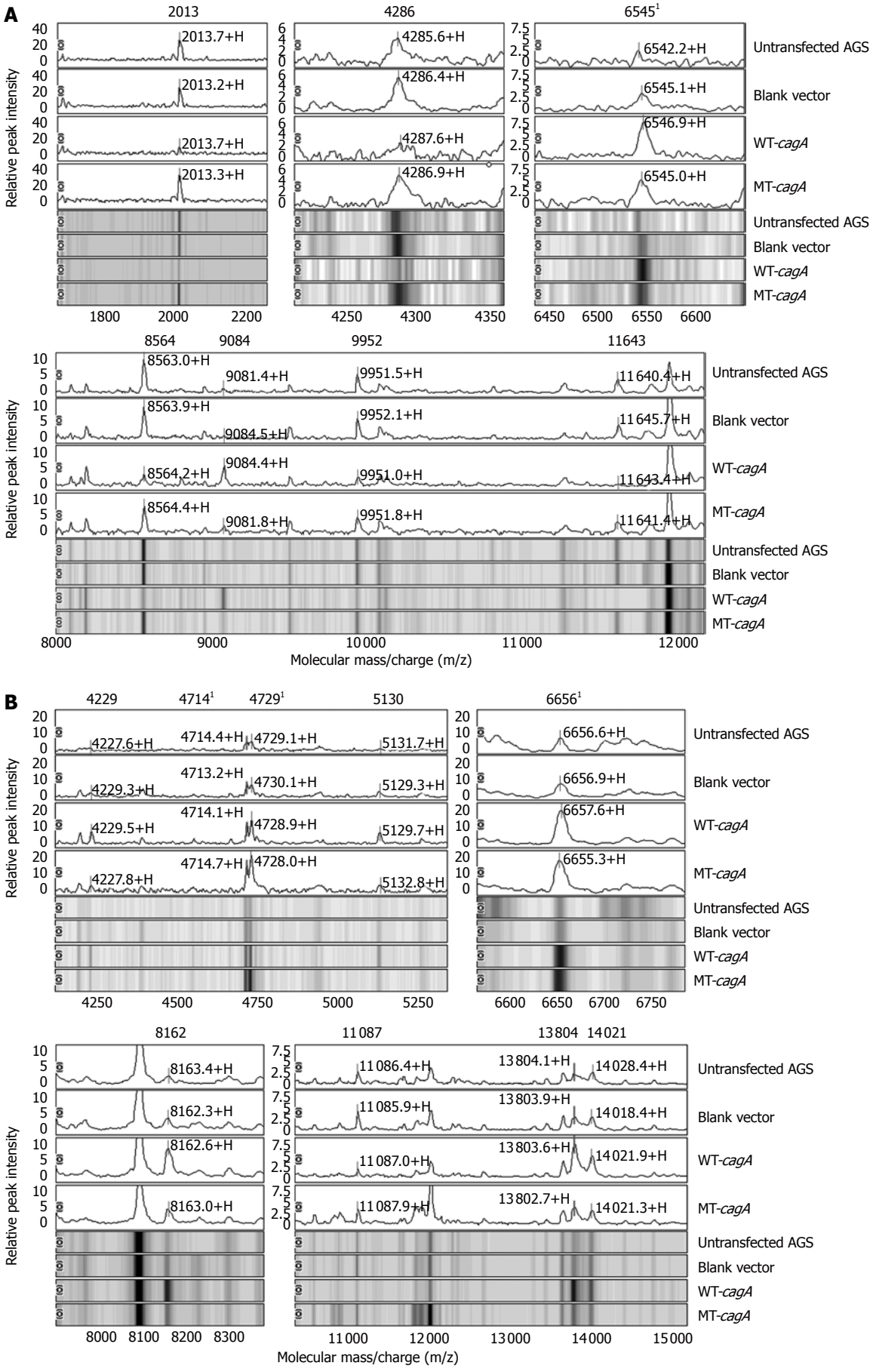


Figure 2 Biomarker proteins with different expression level in WT-*cagA*, phosphorylation site mutant *cagA* (MT-*cagA*) and blank-vector-transfected AGS cells detected by ProteinChip arrays. Cell lysates of each group were analyzed on SAX2 and CM10 Chip surfaces under laser intensity of 180 and sensitivity of 7. One representative spectrum was selected from a quadruplicate set of samples for each group. The top panel in each figure is the spectral view, and the lower panel is the gel view. Peaks with different expression level were marked with m/z values. **A**: Biomarkers detected by SAX2; **B**: Biomarkers detected by CM10. [1]Indicates CagA tyrosine-phosphorylation-independent proteins, which can be induced by both WT CagA and MT CagA (adopted from *Chinese J Cell Biology* 2006; 28: 603-610).

Table 1 Overview of biomarkers' expression level in the 2000-20000 m/z mass range in wild-type *cagA*, phosphorylation site mutant *cagA* and blank vector transfected AGS cells

Array type	m/z	Peak intensity (mean ± SD)			Fold change		Comments
		V	W	M	W *vs* V	M *vs* V	
SAX2	2013	30.39 ± 7.19	8.82 ± 5.37	29.92 ± 9.53	-3.45[a]	-1.02	CagA phosphorylation dependent
	4286	5.08 ± 0.85	2.25 ± 0.70	4.44 ± 0.58	-2.26[a]	-1.14	
	8564	9.73 ± 1.88	3.72 ± 0.42	10.86 ± 1.77	-2.62[a]	1.12	
	9084	2.61 ± 1.35	6.13 ± 1.74	2.75 ± 0.60	2.35[a]	1.05	
	9952	5.22 ± 2.07	2.51 ± 0.95	5.19 ± 1.30	-2.08[a]	-1.01	
	11643	3.39 ± 0.98	1.32 ± 0.86	3.70 ± 1.10	-2.57[a]	1.09	
CM10	4229	4.29 ± 0.57	9.36 ± 0.32	6.13 ± 0.78	2.18[a]	1.43	
	5130	3.59 ± 0.72	6.93 ± 0.61	4.58 ± 0.46	1.93[a]	1.28	
	8162	4.26 ± 0.38	8.88 ± 1.06	4.97 ± 0.24	2.08[a]	1.17	
	11087	3.37 ± 0.75	1.94 ± 0.46	3.19 ± 0.39	-1.74[a]	-1.06	
	13804	2.97 ± 0.79	8.66 ± 0.48	4.14 ± 1.23	2.92[a]	1.39	
	14021	3.06 ± 0.24	5.59 ± 0.41	3.66 ± 1.04	1.83[a]	1.20	
SAX2	6545	3.08 ± 0.88	6.95 ± 1.34	5.53 ± 0.69	2.26[a]	1.80[a]	CagA phosphorylation independent
CM10	4714	10.16 ± 1.26	17.95 ± 3.61	17.31 ± 1.41	1.77[a]	1.70[a]	
	4729	11.11 ± 1.75	20.51 ± 4.02	21.92 ± 2.58	1.80[a]	1.97[a]	
	6656	9.67 ± 2.45	22.83 ± 4.27	21.46 ± 1.92	2.36[a]	2.22[a]	

Biomarkers were selected by these criteria: Statistical *P*-value among sample groups less than 0.05; signal to noise ratio higher than 5.0; peak intensity higher than 1.0. The particular fold change in average peak intensity of wild-type *cagA* transfectants (W) or phosphorylation site mutant *cagA* transfectants (M) was calculated compared with the intensity of blank vector transfectants (V). [a]$P < 0.05$. Statistical *P*-value was calculated with a non-parametrical Mann-Whitney test ($n = 4$).

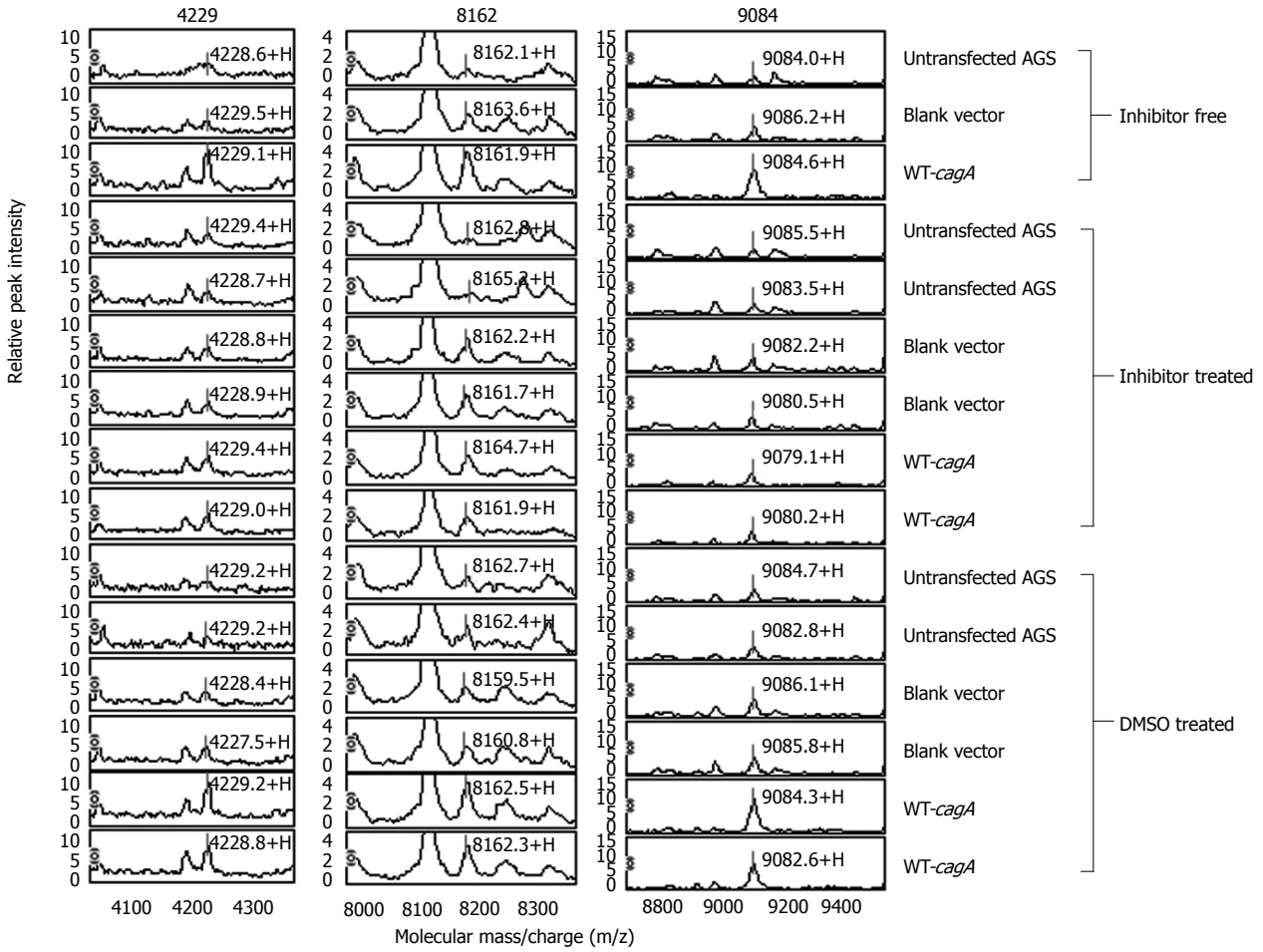


Figure 3 Expression differences of biomarker proteins caused by ERK pathway activation. Cells were transfected with WT-*cagA* or blank vectors with treatment of MEK inhibitor U0126, or its vehicle DMSO. Expression differences of three biomarkers with m/z 4229 (CM10), 8162 (CM10) and 9084 (SAX2) between WT-*cagA* transfectants and vector transfectants appeared under both MEK-inhibitor-free and DMSO-treated conditions, but disappeared under inhibitor-treated conditions.

inhibitor simultaneously to transfection to investigate if the changes in expression of the 16 biomarkers induced by WT-*cagA* disappeared when ERK1/2 was deactivated. The expression levels of three biomarkers with m/z 4229, 8162 and 9084 were found up-regulated significantly in WT-*cagA* transfectants with 2.25-, 1.71- and 1.64-fold changes

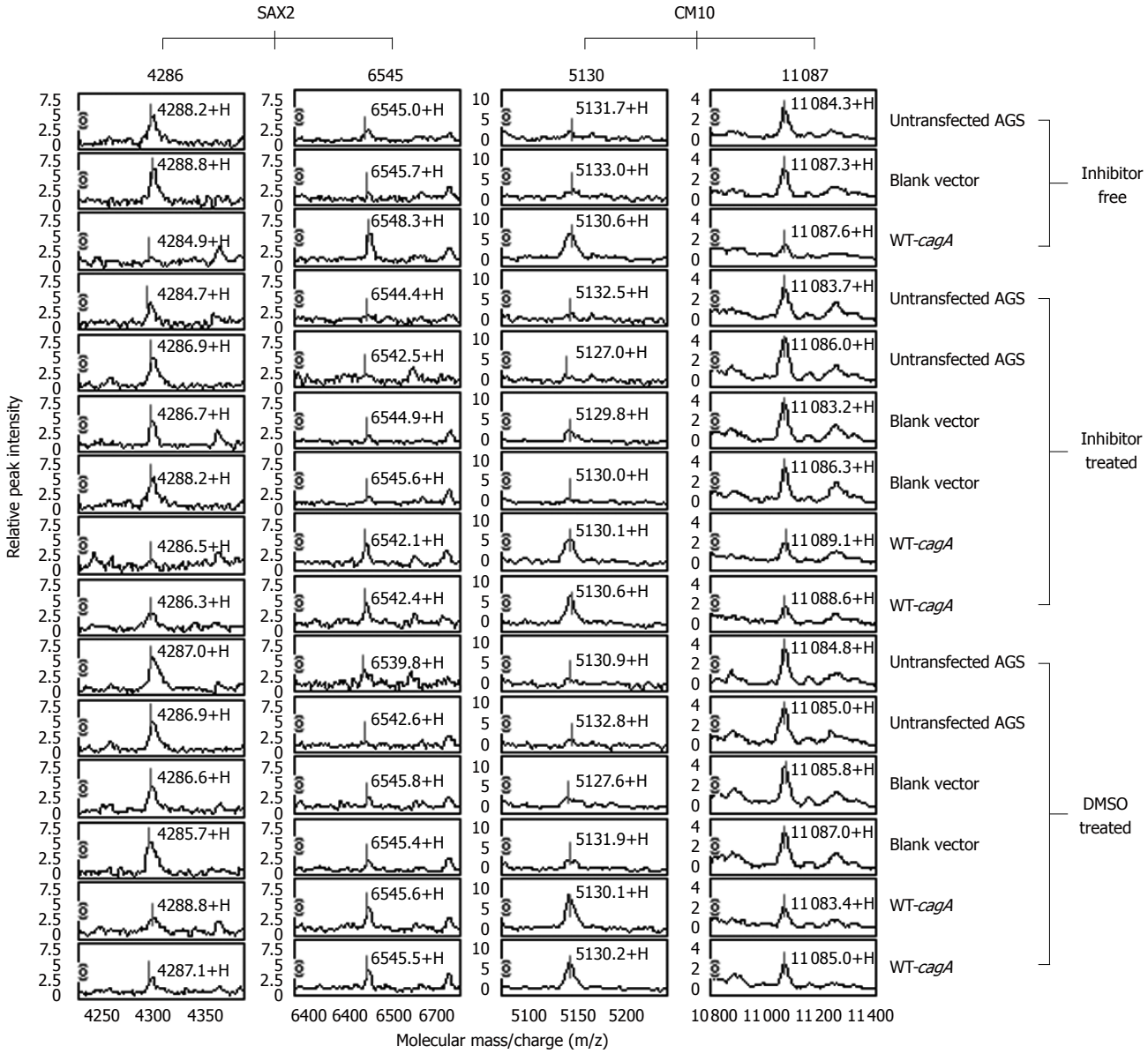


Figure 4 Expression differences of biomarker proteins not caused by ERK pathway activation. Expression differences of the other 13 biomarker proteins between WT-*cagA* transfectants and vector transfectants appeared not only under MEK-inhibitor-free and DMSO-treated conditions, but also under MEK-inhibitor-treated conditions. Spectra of typical biomarker proteins with m/z 4286 (SAX2), 6545 (SAX2), 5130 (CM10) and 11 087 (CM10) are shown in the figure.

compared with vector transfectants under inhibitor-free conditions (Figure 3, panels 1-3). The fold changes were very similar to those in our previous experiment (Figure 2, panels 1-3). In DMSO-treated conditions, they displayed the same expression pattern (Figure 3, panels 10-15) as under inhibitor-free conditions. However, when treated with MEK inhibitor during transfection, the peak intensity of these three proteins in WT-*cagA* transfectants was found not to be significantly different ($P > 0.05$) from that of vector transfectants (Figure 3, panels 4-9). This indicates that the expression differences of biomarkers with m/z 4229, 8162 and 9084 were caused by activation of the ERK/MAPK signaling pathway. The expression differences for the other 13 proteins existed in both ERK1/2-active and -inactive conditions (Figure 4, Table 2). Thus, they were independent of ERK1/2 activation. The biomarkers with m/z 4229, 8162 and 9084 that were identified as ERK1/2 downstream molecules involved in CagA-induced pathogenesis are worthy of further investigation.

Database searching for the three ERK-related biomarkers

A search in the SwissProt/TrEMBL database using the TagIdent tool, along with the pI and mass information was performed to get matching proteins for the three biomarkers with m/z 9084, 4229 and 8162. All together, 22 pieces of matching information were obtained from the database. Seven of these were shown to be proteins related to apoptosis, antimicrobial defense, chemotaxis, proliferation, differentiation and carcinogenesis, which may be closely associated with the ERK/MAPK signaling pathway (Table 3). The existence of these proteins was validated at the protein or transcript level in a series of studies. The other 14 were predicted to be proteins or proteins lacking the references providing insightful

Table 2 Comparison of biomarkers' expression level under mitogen-activated protein kinase kinase (MEK) inhibitor free, treated and DMSO treated conditions

Array type	m/z	Inhibitor free		Inhibitor treated		DMSO treated		Comments
		Fold change (W *vs* V)	*P*-value	Fold change (W+I *vs* V+I)	*P*-value	Fold change (W+D *vs* V+D)	*P*-value	
SAX2	9084	1.64	0.021	1.00	0.827	2.21	0.021	Caused by ERK1/2
CM10	4229	2.25	0.021	1.07	1.000	2.44	0.021	activation
	8162	1.71	0.021	-1.15	0.149	1.81	0.021	
SAX2	2013	-1.99	0.021	1.16	0.149	1.03	0.773	Have no relationship
	4286	-3.50	0.021	-2.14	0.021	-1.95	0.021	with ERK1/2
	6545	2.86	0.021	2.39	0.021	2.00	0.021	activation
	8564	-3.05	0.021	-2.96	0.021	-1.85	0.021	
	9952	-3.35	0.021	-2.87	0.021	-2.62	0.021	
	11643	-1.71	0.021	-2.49	0.021	-2.38	0.021	
CM10	4714	1.89	0.021	1.68	0.043	1.74	0.021	
	4729	1.99	0.021	1.72	0.021	1.86	0.021	
	5130	1.67	0.021	1.66	0.021	2.91	0.021	
	6656	2.12	0.021	2.31	0.021	2.44	0.021	
	11087	-1.72	0.021	-1.85	0.021	-1.52	0.021	
	13804	2.23	0.021	2.56	0.021	2.60	0.021	
	14021	1.98	0.021	1.79	0.021	1.61	0.021	

Biomarkers listed in top three lanes were identified as biomarkers induced by extracellular signal-regulated kinase 1/2 (ERK1/2) activation. Columns 3, 5, 7 give fold changes in peak intensity in wild-type cagA transfectants (W) compared with vector transfectants (V) as reference under MEK inhibitor U0126 free, MEK inhibitor U0126 treated (I) and DMSO treated (D) conditions. Statistical *P*-value were calculated using a non-parametrical Mann-Whitney test (n = 4).

Table 3 Swiss-Prot and TrEMBL database searching results of the three ERK related biomarkers

Biomarker (Mw)	Meaningful matching information in Swiss-Prot and TrEMBL database			Comments
	Protein Name	Mw	pI	
9084 (SAX2)	BIM-alpha3 (BCL2-like 11 transcript variant 10, chain 1-79)	9081	5.42	Over-expression of BIM-alpha3 results in cell apoptosis
	EP2E (chain 1-80)	9091	7.55	Part of the protein sequences homologous to β-defensins, a family of antimicrobial peptides.
	UPF0197 protein (C11orf10 chain 1-79)	9079	5.58	Expressed in CD34+ hematopoietic stem/progenitor cells
4229 (CM10)	Chromogranin-A Precursor (ER-37, chain 420-456)	4233	4.59	Neuroendocrine and endocrine secretory granules, considered as biomarkers in some human cancers
8162 (CM10)	Small-inducible cytokine B6 Precursor (chain 40-114)	8162	9.74	Functional characterization as chemokines, chemotactic for neutrophil granulocytes
	Putative microRNA host gene protein 1 (chain 1-70)	8163	9.25	Highly expressed in B-cell lymphoma and lung cancer and enhanced cell proliferation
	PNAS-145 (Chain: 1-68)	8161	11.24	Human acute promyelocytic leukemia cell line NB4's apoptosis/differentiation related genes

Searching was carried out using TagIdent tool with mass and the pI information (0-9.0 for SAX2, 4.0-14.0 for CM10).

functional information. Therefore, the three biomarkers with m/z 9084, 4229 and 8162 have great potential to be identified as cancer-associated proteins in future research.

DISCUSSION

Translocation of CagA protein into gastric epithelial cells through the type Ⅳ secretion system is considered to be one of the most important processes in the progression of *H pylori* diseases. In this host-bacterial interaction period, many host proteins and signaling pathways are implicated[33]. In order to gain an overall view into the host response to CagA, the innovative proteomic technology SELDI-ProteinChip platform was employed to investigate the expression changes in gastric epithelial AGS cells. The strongpoint of this technology is that it can detect proteins in fmol quantities, and it allows fast, high-throughput analysis, especially in the low-molecular-weight range, which is essential for biomedical research, and cannot be easily achieved by conventional methods. Following this method, 16 biomarkers have been identified in our previous study as being involved in CagA-host cell interactions, and 12 of these are dependent on CagA phosphorylation. This result provides a primary view of CagA-induced pathological changes at the protein level.

To further investigate the role of CagA-phosphorylation-associated functions in gastric carcinogenesis, we focused our interest on the role of the ERK signaling pathway. It has been verified by clinical pathological tests that the level of phosphorylated ERK in gastric cancer tissue is higher than that in the gastric mucous membrane. The level is also higher in cancer tissue of *H pylori*-positive gastric cancer patients than that in *H pylori*-negative patients[34]. Besides, it has been reported that *cagA*-positive *H pylori* is more powerful than *cagA*-negative strains for ERK activation[35]. Thus, ERK/MAPK pathway activation and subsequent cell proliferation, differentiation, as well as morphological changes, are among the most important pathogenic factors

in CagA-induced diseases. However, this complicated process includes multi-level regulation that has rarely been understood. In the present study, we tried to investigate if the expression changes of the 16 host proteins induced by CagA have some relationship with the activation of the ERK pathway. The phosphorylation level of ERK1/2 in WT-*cagA* transfectants was confirmed to be much higher than that in vector transfectants and blank control cells. MEK inhibitor U0126 that specifically inhibits ERK1/2 phosphorylation was employed to eliminate the changes induced by ERK activation during the transfection of WT-*cagA*. The relationship of the 16 biomarkers and ERK pathway can be determined by comparing their expression levels under ERK-inhibited and ERK-activated conditions. On the other hand, protein profiles indicated that protein expression levels were influenced by DMSO as the solvent of U0126. Therefore, we first compared profiles of WT-*cagA* transfectants and vector transfectants under U0126-free conditions, U0126 treatment, and DMSO treatment. Only the proteins exhibited similar expression differences between WT-*cagA* transfectants and vector transfectants under both U0126 free and DMSO treated conditions, but they exhibited no expression differences under the U0126 treated condition, and thus were considered to be biomarkers that related to ERK activation. In this manner, among the 16 biomarkers, three with m/z 4229, 8162 and 9084 were identified as ERK pathway downstream proteins related to CagA pathogenesis. These three biomarkers will be characterized in our future research.

Most biomarkers discovered so far are peptides or small proteins with low abundance. They are probably metabolic products, abnormally cleaved proteins, modified proteins, neurokinins or cytokines that exist in disease states. The biomarkers detected in our study are also likely small molecules of this kind, which can hardly be detected by traditional methods, but are essential for associated research. While the SELDI-ProteinChip platform is very rapid for protein profiling, it does not allow direct purification and functional identification. A search in the SwissProt/TrEMBL database using the TagIdent tool with pI and mass information was performed to obtain matched information for each biomarker. The search results suggested that these three biomarkers are probably peptides of the following type: β-defensin homologs, which might be involved in host defense to bacterial infection; BCL2-like protein, which might function as an apoptosis inhibitory protein; chemokines; and tumor-related proteins, which might be responsible for cell proliferation and differentiation induced by ERK/MAPK pathway activation, and contribute to CagA-induced carcinogenesis in the gastric epithelium. The matching information provides helpful references for obtaining corresponding antibody which will be employed in future biomarker identification. The biochemical properties provided by the selective ProteinChip surfaces and SELDI-TOF-MS for each biomarker are also useful for the purification process. Further purification and identification will elucidate the function of related small molecules in CagA pathogenesis, and may therefore discover new therapeutic targets.

COMMENTS

Background

H pylori infection is an important causative factor of gastritis, peptic ulcer and gastric cancer. CagA, the major virulence factor of type I *H pylori*, is injected by the bacterium into gastric epithelial cells, and has been shown by epidemiological and experimental studies to be closely associated with the development of *H pylori*-induced diseases, especially gastric cancer. However, the precise role of CagA in cell function after *H pylori* infection remains unclear.

Research frontiers

CagA is translocated into host cells through a type Ⅳ secretion system of *H pylori*. After tyrosine phosphorylation at the C terminus, CagA strongly interferes with signal transduction, which results in cell proliferation and differentiation. Various studies have indicated a relationship between CagA, ERK/MAPK pathway activation and gastric cancer. In this study, a proteomic approach was employed to detect host cell proteins related to CagA-induced ERK pathway activation.

Innovations and breakthroughs

A new proteomic approach based on ProteinChip array and SELDI-TOF-MS was employed to investigate the global protein expression changes in gastric epithelial cells induced by CagA. Three biomarkers were further identified as downstream molecules of ERK1/2 in the ERK/MAPK signaling pathway. Due to the high sensitivity and resolution in the low-molecular-weight range of SELDI-ProteinChip technology, biomarkers discovered in this study are mainly low-mass and/or low-abundance disease-related proteins, which are difficult to detect by traditional methods.

Applications

These results provide new targets for further understanding the biological function and pathogenesis of CagA, as well as new therapeutic targets.

Terminology

CagA: cytotoxin-associated gene A protein encoded by cytotoxin-associated gene pathogenicity island is the major virulence factor of type I *H pylori*. ERK: extracellular signal-regulated kinase. ERK pathway is one of the major three members of the MAPK signaling pathways. ERK has been reported as a key molecule in carcinogenesis in various cell types. ProteinChip array: the surface of array selectively retains proteins from biological samples according to their biochemical properties, and can be analyzed directly by MS. SELDI-TOF-MS: surface-enhanced laser desorption/ionization time-of-flight MS detects proteins in fmol quantities, and allows fast, high-throughput analysis, especially in the low-molecular-weight range.

Peer review

This is a state-of-the-art, well-written paper that has used a proteomic approach to understand CagA protein biology as it relates to gastric cancer.

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RAPID COMMUNICATION

Artificial neural networks in the recognition of the presence of thyroid disease in patients with atrophic body gastritis

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Abstract

AIM: To investigate the role of artificial neural networks in predicting the presence of thyroid disease in atrophic body gastritis patients.

METHODS: A dataset of 29 input variables of 253 atrophic body gastritis patients was applied to artificial neural networks (ANNs) using a data optimisation procedure (standard ANNs, T&T-IS protocol, TWIST protocol). The target variable was the presence of thyroid disease.

RESULTS: Standard ANNs obtained a mean accuracy of 64.4% with a sensitivity of 69% and a specificity of 59.8% in recognizing atrophic body gastritis patients with thyroid disease. The optimization procedures (T&T-IS and TWIST protocol) improved the performance of the recognition task yielding a mean accuracy, sensitivity and specificity of 74.7% and 75.8%, 78.8% and 81.8%, and 70.5% and 69.9%, respectively. The increase of sensitivity of the TWIST protocol was statistically significant compared to T&T-IS.

CONCLUSION: This study suggests that artificial neural networks may be taken into consideration as a potential clinical decision-support tool for identifying ABG patients at risk for harbouring an unknown thyroid disease and thus requiring diagnostic work-up of their thyroid status.

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Key words: Atrophic body gastritis; Thyroid disease; Artificial neural networks

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INTRODUCTION

Atrophic body gastritis (ABG) is characterized by loss of the oxyntic glands and lack of production of chlorhydric acid and intrinsic factor[1], and is epidemiologically and biologically linked to the development of the intestinal-type gastric adenocarcinoma and gastric carcinoid type I [2]. ABG often presents clinically with pernicious anaemia (PA)[3], but may present also with iron deficiency anaemia and/or long-standing dyspepsia[4,5].

The association of ABG with thyroid disorders (TD) has firstly been described about 40 years ago[7-9], and the term "thyrogastric disease" has been used to define the presence of thyroid autoantibodies and/or autoimmune thyroid disease in patients with PA[8,10-12]. These older studies assessed the association between PA and thyroiditis on the basis of gastric and/or thyroid autoantibodies, and found an association ranging from 2.6%-32%[9-12].

Undiagnosed TD may lead to complications related to subclinical hypo- or hyperthyroidism, which may be prevented by an early diagnosis of TD[13]. The diagnosis of TD is generally based on clinical history, assessment of thyroid hormones and autoantibodies as well as ultrasonographic evaluation of the thyroid gland[14]. Albeit, these procedures are non-invasive, a complete diagnostic work-up of TD is time- and cost-consuming. Thus, reliable tools able to select those ABG patients at high risk for harbouring an undiagnosed TD may be useful.

Decision-support systems, based on conventional statistical methods made their entry into medicine several years ago[15], and efforts to improve predictive and prognostic performance of these systems have led to the application of artificial neural networks (ANN) as tools for clinical decision-making[16-18]. ANN are highly flexible

computerized mathematical models for understanding and predicting complex and chaotic dynamics in complex biological systems, and have been effectively used to solve non-linear problems related to diagnostic or prognostic queries[16,19]. Thus, ANN would appear to be a promising tool for clinical decision-making and have been applied in various areas of medical research[20-22]. In particular, this tool has been previously applied with a high accuracy for predicting atrophic body gastritis on the basis of clinical and serological parameters in patients with anaemia and/or dyspepsia[23]. To our knowledge, studies applying advanced statistical methods in patients with ABG and TD have not yet been performed. This study, therefore, aimed to investigate the role of ANN and linear discriminate analyses (LDA) in predicting the presence of TD in ABG patients.

MATERIALS AND METHODS

Patients

This study was carried out using a database comprising data from 253 ABG patients (185 females, median age 54 [17-83] years). Of these ABG patients, 123 (48.6%) had pernicious anaemia. In all patients, the presence or absence of TD was evaluated by biochemistry, ultrasonography and endocrinological evaluation at a single tertiary centre. TD was present in 135 (53.4%), and 118 (46.6%) had a healthy thyroid gland. In detail, of the 135 ABG patients with TD, 102 (75.6%) had an autoimmune TD and the remaining 33 (24.4%) had non-autoimmune thyroid disorders, such as a multinodular goitre (n = 16) or a previously diagnosed subacute thyroiditis (n = 10). In addition, 7 patients had undergone complete thyroidectomy prior to the diagnosis of ABG: 3 for thyroid cancer, 3 for large multinodular goitre and 1 for autoimmune thyroiditis.

For each of these 253 patients at the time of diagnosis of ABG, a structured questionnaire was filled out, composed of 29 items concerning anagraphical, life style, family and clinical history, biochemical and histological variables.

Criteria for the diagnosis of ABG: The diagnosis of ABG was based on the presence of fasting gastrin above upper normal values, and histological confirmation of gastric body mucosal atrophy, as previously described[24, 25]. Briefly, all patients underwent gastroscopy with standardized biopsy sampling from the antrum (n = 3), body (n = 3) for conventional histopathological examination[24,25]. The degree of gastritis was assessed according to the updated Sydney System[26]. Atrophy of the body and antral mucosa was defined as focal or complete replacement of oxyntic or pyloric glands by metaplastic pyloric or intestinal glands, respectively[24,25]. Furthermore, all patients underwent serological studies: fasting gastrin levels were evaluated by means of a specific radioimmunoassay (RIA) using polyclonal antibody No. 4562[24,25]. Pepsinogen I levels were measured using a commercial RIA kit (Pepsik, Sorin, Saluggia, Italy)[25]. Antibodies against anti-parietal cells were assayed using a commercial kit (Autostat, Cogent Diagnostic Ltd, Edinburgh, UK)[24,25].

Criteria for diagnosis of thyroid disease: The thyroid status of ABG patients was evaluated on the basis of clinical history, biochemical and ultrasonographic evaluation. Thyroid hormones and thyroid autoantibodies in serum were determined by commercial kits: free triiodothyronine and free thyroxin levels were assayed by radioimmunoassay (Ares-Serono, Milan, Italy); basal thyrotropin levels were assayed by immunoradiometric assay (Radim Techland, Liege, Belgium); antiperoxidase antibodies were measured by a radioligand assay (Radim Techland, Liege, Belgium)[27]. Thyroid gland size, echogenicity of the parenchyma, and nodular lesions were evaluated by ultrasonographic examination. The diagnosis of autoimmune thyroiditis was based on the presence of antiperoxidase antibodies (antibody titres stably > 200 U/mL in at least two separate measurements performed at least 6 mo apart) and characteristic ultrasound features (i.e. non-homogeneous pattern with diffuse reduction of echogenicity) according to Rago *et al*[14] in presence, but even in absence of mild or overt hypothyroidism[28]. Non-autoimmune thyroid disease (NATD) was assessed on the basis of the presence of ultrasonographic thyroid abnormalities (increased thyroid volume > 25 mL, with the presence of one or more nodules) and the definite absence of antiperoxidase antibodies. Only patients with definite normal morphofunctional and immunological parameters were assumed to have a healthy thyroid gland.

All patients gave written informed consent to the study, which was approved by the local Ethics Committee.

Study design and statistical evaluation

As shown in Table 1, the study was performed on the dataset of 29 input variables of the 253 ABG patients, of which five were continuous, whereas the remaining were dichotomous, as previously described[23]. Biochemical and ultrasonographic data on the diagnosis of TD were not included in the data set. The presence or absence of TD was considered as target variable.

Artificial neural networks (ANN): As shown in Figure 1, the sample of ABG patients was randomly subdivided several times into two equal and balanced sub-samples of subjects with and without TD; one for the training phase (testing) and one for the prediction phase (training). During the training phase, a fixed ANN model is chosen and submitted to the training sub-set in order to learn the linear and non-linear relations existing between all the variables of the original data set and between the input variables and the target variables, performing, in this way, a classification task, i.e., the correct recognition of ABG patients with and without TD. At the end of the training phase, the ANN models showing the best performance on the classification task were submitted to the second sub-sample of patients (the testing set), not previously exposed to the networks, in order to ensure an unbiased evaluation of the classification capability of the ANN. During the so-called "testing phase", the target variable (presence/absence of TD) has been excluded and the networks made its own evaluation, based on the previously carried out training. In the cross validation protocol, the pairs of sub-set are inverted during the elaboration procedure: each sub-set is used once for the

Table 1 Dataset of the 253 patients with atrophic body gastritis: original 29 input variables used by the standard artificial neural network (ANN) and input variables selected as the most informative by the T&T and IS System and the TWIST Protocol during the input data optimisation procedures

Input variables	Standard ANN	T&T and IS system	TWIST protocol
Positive clinical history for peptic ulcer	X	X	X
Active *H pylori* infection	X	X	X
Male	X	X	
Female	X	X	X
Age at diagnosis of ABG[1]	X	X	
ABG without associated neoplastic lesions	X		
ABG with associated neoplastic gastric lesions	X	X	X
Referred for anemia	X	X	
Referred for long-standing dispepsia	X		
Referred for dermatological disorders	X	X	X
Referred for endocrinological disorders	X		X
Referred for neurological disorders	X	X	
Presence of metaplastic atrophy	X	X	X
Presence of multifocal atrophic gastritis	X	X	
Actual smoker	X	X	
Previous smoker	X	X	
Never smoked	X	X	
Positive familiy history for gastric cancer	X		
Positive family history for peptic ulcer	X	X	
Presence of extrathyroidal autoimmune diseases	X	X	X
Presence of extragastric neoplasms	X	X	X
Presence of pernicious anemia	X	X	X
Presence of iron deficiency anemia	X	X	X
Absence of anemia	X	X	X
Hemoglobin[1]	X	X	X
Mean corpuscular volume[1]	X	X	X
Fasting gastrin[1]	X		
Pepsinogen I[1]	X	X	X
Presence of parietal cell antibodies	X	X	X
Total number of input variables used	29	24	16

X = selected variables; [1]continous variables.

training and once for the testing, this allowing to obtain a non-polarised classification tool.

In order to reduce the number of input variables, selecting those most informative to predict the output, the T&T system, the IS system, as well as the TWIST protocol were used (Table 1). The first system has been developed to solve the problem of establishing the size and assessing the quality of the training and testing sets for a more effective data processing through the ANN models. T&T is a data resampling technique, based on an evolutionary algorithm developed by the Semeion Research Center, the Genetic Doping Algorithm (GenD), able to split the source sample into two or more sub-samples, each one with a similar probability density function. The IS system is an evolutionary wrapper system, also based on the evolutionary algorithm GenD, able to reduce the amount of data, while conserving the largest amount of information available in the dataset. In the TWIST protocol, the T&T and IS systems run in a parallel way to reach a suitable representation of variables and the optimal sample size when dealing with complex and non-linear problems[9]. The software used for the ANN analysis has been developed and were applied by the Semeion Research Center, Rome, Italy.

Linear discriminant analyses (LDA): As shown in Figure 1, the same database was analyzed applying a statistical model based on the assumption of linear correlations between variables, the LDA. In order to optimize the predictive ability, different models were assessed and, as for the ANN procedure, the samples were randomly divided into several sub-samples for the training and testing phases. For the analysis of LDA, the SAS version 6.04 (SAS Institute, Cary, NC, USA) using stepwise procedure was employed. This was performed by the Semeion Research Center, Rome, Italy.

RESULTS

LDA and standard ANN

As shown in Table 2, the predictive performance of LDA in discriminating ABG patients with and without TD reached a mean accuracy of 59%, predicting correctly 61% of the ABG patients with TD (sensitivity) and 58% patients with a healthy thyroid gland (specificity). In this model, 82 out of the 135 ABG patients with TD and 69 of the 118 ABG patients without TD were correctly identified. In contrast, the standard ANN model, which used all the 29 input variables, recognized correctly 93 ABG patients with TD and 70 without TD, yielding a mean accuracy, a sensitivity and a specificity of 64%, 69%, and 60%, respectively. Thus, as far as regards the specificity, the predictive performance of LDA and standard ANN were similar, because LDA missed 49 patients and standard

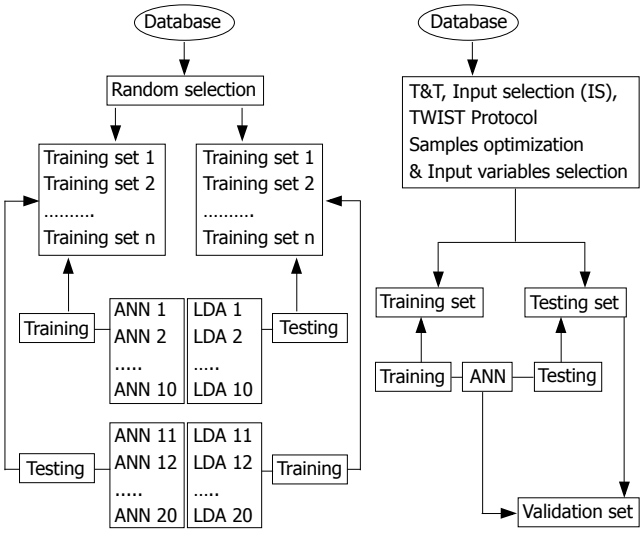


Figure 1 Application protocols of artificial neural networks and linear discriminant analyses.

ANN 48 (P = 1.00). As far as concerns the sensitivity, i.e. the performance in predicting ABG patients with TD, also in this case, the predictive ability of the standard ANN model was similar to that of LDA with 53 and 42 misclassified patients, respectively (P = 0.20).

Data optimization by T&T and IS systems

In a first step of input data optimization, the sequential application of the T&T and IS systems selected as the most informative 24 of the original 29 independent variables (Table 1). The ANN run with these 24 input variables made less classification errors and thus improved the predictive performance compared to the previous ANN performed on all 29 variables, matching correctly 106 ABG patients with TD and 83 without TD and thus yielding a mean predictive accuracy, a sensitivity and a specificity of 75%, 78%, and 70%, respectively (Table 2).

This ANN model with 24 variables misclassified 35 ABG patients without TD, but the statistical significance with respect to LDA and standard ANN was not reached. In contrast, the predictive performance in identifying correctly ABG patients with TD improved significantly by using the optimized ANN model with 24 variables ($P < 0.01$) with respect to LDA, reducing the misclassified ABG patients from 49 (LDA) to 29 (ANN 24 variables).

Data optimization by TWIST protocol

Finally, the application of the TWIST protocol selected as the most informative 16 of the original 29 variables (Table 1). This ANN model reached a better predictive performance compared to the other ANN models, correctly recognizing 82% of the ABG patients with associated TD and 70% of the ABG patients with a healthy thyroid gland; in this case the mean predictive accuracy was 76%, thus outperforming the previous ANN models as well as the traditional linear models in the recognition task. Indeed, the predictive performance in identifying correctly ABG patients with TD improved significantly by using the optimized ANN model with 16 variables ($P < 0.001$) with respect to LDA, reducing the misclassified ABG patients from 49 (LDA) to 25 (ANN 16 variables). On the other hand, with respect to standard ANN, the predictive performance of the optimized ANN model with 16 variables was improved (P = 0.02).

Table 2 Predictive performance of linear discriminant analyses (LDA) and artificial neural networks (ANN) in recognizing atrophic body gastritis patients with and without thyroid disease (%)

	Sensitivity	Specificity	Mean accuracy
LDA	60.8	58.1	59.4
Standard ANN (29 variables)	69.0	59.8	64.4
T&T and IS (24 variables)	78.8[b]	70.5	74.7
TWIST protocol (16 variables)	81.8[d]	69.9	75.8

[b]$P < 0.01$ *vs* LDA; [d]$P < 0.001$ *vs* LDA and *vs* standard ANN $P < 0.05$.

DISCUSSION

The results emerging from this study suggest that artificial neural networks are able to predict, with a good accuracy, the presence of TD in ABG patients, by using clinical and gastric biochemical and histological variables. Indeed, the optimised ANN yielded an accuracy of 76%, correctly identifying 82% of ABG patients with TD.

Indeed, the association between ABG and TD is strong, because, as confirmed by the findings of this study, more than half of patients with ABG have an associated TD. Previous studies found an important association up to 30% between pernicious anaemia and TD on the basis of gastric and/or thyroid autoantibodies[30,31] and a more recent report assessed the coexistence of ABG and TD on more updated diagnostic criteria[27]. Undiagnosed TD may lead to complications related to subclinical hypo- or hyperthyroidism, which may be prevented by an early diagnosis of TD[13]. Albeit, the diagnostic procedures are generally non-invasive[14] and thus easily accepted by the patients, a complete diagnostic work-up of TD is time and cost consuming. Thus, a computer-based decision-support system, able to select, with an acceptable predictive accuracy, those patients with a high probability of having TD, would enable the thyroid investigations to be better addressed.

Generally, the real databases are composed by a large number of variables that, only apparently, provide the largest possible amount of information. In fact, data collection is performed by trying to include in the database all variables that can have a connection with the problem under investigation; but some of these variables may contain redundant information, which is included also in other variables, or confused information (noise), or may not even contain any significant information at all and be completely irrelevant. If inserted into the dataset, these variables can cause a greater difficulty for the ANN to learn data correctly. Indeed, in this study complex "intelligent" data optimization procedures based on evolutionary algorithms were applied, which permitted to reduce the amount of data, while conserving the largest amount of information available in the dataset, to reach a suitable representation of variables and the

optimal sample size when dealing with complex and non-linear problems[29]. This approach, in fact, increased the predictive accuracy as well as the specificity and the sensitivity compared to models performed on non-optimized datasets. Indeed, the reduction in the number of input variables from the initial 29 to 16 led to an increase of the accuracy and the sensitivity of ANN from 64% to 76% and from 69% to 82%, respectively.

It is worthwhile to mention that among the most informative variables for the prediction of TD in ABG patients, Pepsinogen I , parietal cell antibodies, female gender and the type of anaemia were included. This finding is not surprising, because all these features have been reported to be related to ABG[2,13,25]. Among variables with the highest predictive power for the presence of TD in ABG patients, the data optimization procedures selected also the presence of active *H pylori* infection and the positive clinical history for peptic ulcer, which is also related to *H pylori* infection. This finding is quite interesting, since some authors suggest that the coexistence of ABG and TD may be attributable to common organ-specific antigens that occur as a result of these organs sharing common embryonic origin[32]. However, in general, the presence of a statistical relationship between a predictor variable and the outcome alone does not imply causality[33]. This is even more the case for ANNs-based models, which process data in a non-linear way, and the network logic of prediction cannot be broken down into simple elements of clinical reasoning[34,35]. Thus, the variables with the most predictive power selected by the advanced statistical systems should not be viewed as independent predictive variables as perceived by a clinician[33,34].

We are aware that the current study had some limits. For the clinical applicability of computer-based decision support systems and their acceptance by physicians, it is necessary to offer a system which is simple, with entry of data that requires little time, thus using a limited number of variables which are readily available to the clinician. In our final optimized ANN model, 16 input variables were selected as most informative for optimizing the prediction of ABG patients with TD. This large amount of input data may thus be a limit for an eventual clinical applicability of this system since the collection of many input variables may be considered time-consuming and scarcely attractive as a clinical decision tool. In any case, this study did not want to emphasize that advanced statistical decision support systems should replace or substitute experienced clinicians, but to underline that, these systems should be viewed as a potential decision aid in order to better address investigations to save costs and to use resources when effectively necessary, as described in other fields of medical settings[36] .

Moreover, we are aware that external validation has not yet been carried out and that, at this stage, the utility and cost benefit of artificial neural networks in recognizing thyroid disease in ABG, as opposed for example to performing thyroid hormones and thyroid autoantibodies and/or thyroid ultrasonographic evaluation, needs to be determined.

In conclusion, this study suggests that artificial neural networks may be taken into consideration as a potential clinical decision-support tool for identifying those ABG patients at risk for harbouring unknown TD and thus requiring diagnostic work-up of their thyroid status.

COMMENTS

Background

Atrophic body gastritis (ABG) is characterized by loss of the oxyntic glands and lack of production of chlorhydric acid and intrinsic factor. The association of ABG with thyroid disorders (TD) has firstly been described about 40 years ago, and the term "thyrogastric disease" has been used to define the presence of thyroid autoantibodies and/or autoimmune thyroid disease in patients with ABG, for these reasons thyroid evaluation should be considered in these patients.

Research frontiers

Decision-support systems, based on conventional statistical methods made their entry into medicine several years ago, and efforts to improve predictive and prognostic performance of these systems have led to the application of artificial neural networks (ANN) as tools for clinical decision-making. In particular, this tool has been previously applied with a high accuracy for predicting atrophic body gastritis on the basis of clinical and serological parameters in patients with anaemia and/or dyspepsia.

Innovations

In this article, artificial neural networks are used to investigate their ability in predicting the presence of TD in ABG patients and to evaluate their usefulness in selecting atrophic body gastritis at high risk for harbouring an undiagnosed thyroid disease.

Application

This article suggests that artificial neural networks are able to predict, with a good accuracy the presence of TD in ABG patients, by using clinical, and gastric biochemical and histological variables. Indeed, the optimised ANN yielded an accuracy of 76%, correctly identifying 82% of ABG patients with TD.

Peer review

The paper seems innovative since, to our knowledge, studies applying advanced statistical methods in patients with ABG and TD have not yet been performed and the obtained results clearly indicated the potential use of this tool in ABG patients at risk for harbouring an unknown thyroid disease and thus requiring diagnostic work-up of their thyroid status.

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RAPID COMMUNICATION

Biliary brush cytology: Factors associated with positive yields on biliary brush cytology

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Abstract

AIM: To evaluate the yield of brushing biliary strictures and the factors associated with a positive result in biliary strictures.

METHODS: Data on all consecutive patients (01/02 -10/05) who were identified to have a biliary stricture and who underwent biliary brush cytology were collected. The yield of positive biliary brush cytology was evaluated and compared to results with the gold standard for diagnosis (defined as either definitive surgical histology or clinical course). Additionally, associated factors of positive results including stricture location, gender, age, mass size, length of stricture, and dilatation prior to brushing cytology were assessed.

RESULTS: From 199 patients who had brushing cytology samples (10 patients were excluded due to lack of gold standard diagnosis), 77 patients had positive brushing cytology (yield 41%). Variables associated with positive cytology brushing on initial endoscopic retrograde cholangiography were age 1.02 (1.00-1.05), mass size > 1 cm 2.22 (1.01-4.89) and length of stricture > 1 cm 3.49 (1.18-10.2). The sensitivity of biliary brushing was 61%, its specificity 98%, the positive predictive value reached 99%, and the negative predictive value was 57%.

CONCLUSION: Our results revealed a 41% positive yield from brushing cytology. The sensitivity of biliary brushing cytology in our center was 61% and the specificity was 98%. Predictors of positive yield include older age, mass size > 1 cm, and stricture length of > 1 cm.

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Key words: Biliary; Endoscopic retrograde cholangiopancreatography; Pancreas; Gallbladder; Ampulla

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INTRODUCTION

Biliary duct strictures are most often caused by neoplastic or inflammatory processes involving the biliary tree, pancreas, gallbladder, or ampulla. The use of endobiliary brush cytology to definitively diagnose malignant biliary strictures has been shown to be useful during endoscopic retrograde cholangiography (ERC). However, the diagnostic yield of this technique remains low, ranging from 30% to 80%[1-14] depending on the literature reviewed. Conservative management with biliary drainage is used in many patients with malignant tumors, most of whom are not candidates for curative resection due to locally advanced or metastatic disease. In such patients, an accurate tissue diagnosis may help in developing a concept for further management[3,4,15,16].

Cytological techniques have become the initial diagnostic modality in many cases since biliary lesions are not always readily accessible to biopsy[4,6,7,17]. Percutaneous radiologically guided fine needle aspiration (FNA), although a very accurate technique, is operator dependent and requires a sufficiently distinct mass lesion for targeting. Additionally, in patients who are surgical candidates 'seeding' of the needle tract has been a concern. Brush cytology performed at ERC, in contrast, has a low complication rate and allows sampling from most sites within the biliary duct systems[4,6,7,17].

The objective of this study was to assess the accuracy of brush cytology in biliary strictures and to assess the factors influencing results over a 3.7 years period from January 2002 to September 2005.

MATERIALS AND METHODS

From a total of 2500 endoscopic retrograde cholangiopancreatography performed between January 2002 and September 2005 at our center (St. Paul's Hospital, Vancouver), all biliary brush cytology findings were reviewed. This hospital serves as a tertiary referral center for the management of biliary diseases, and clinicians obtained follow up on all their patients. Two

hundred and ninety nine specimens were received, from one hundred and ninety nine patients in the review period. For inclusion in the study, patients had to have a definite final benign or malignant diagnosis based either on histological or cytological sampling or by clinical outcome. The latter was obtained by case record review. A total of ten patients (3.3%) were excluded from performance characteristics of endoscopic retrograde cholangiopancreatography (ERCP) brush cytology due to lack of gold standard for diagnosis.

Ethics approval for the chart review was performed through St. Paul's Hospital, University of British Columbia, Vancouver, Canada.

The specimens were obtained at ERCP with a Cytomax2, single use, 8 french, 2.5 cm long double lumen Wilson-Cook cytology brush (#4900 Benthania, Station Road, Winston-Salem, NC 27105). Fluoroscopic guidance ensured that the brush was within the stricture. Between ten and fifteen "in and out" passes with the brush was routinely used to ensure an adequate sample, and then the entire brush excised into formalin or saline and sent to the laboratory. Routine cytospin preparations were prepared and stained according to Papanicolaou's method. Samples were classified cytologically as follows: Class 1: Benign; Class 2: Reactive; Class 3: Suspicious for malignancy; Class 4: Cells with cancer morphology recommend biopsy (likely malignant but rarely can be reactive); Class 5: Malignant (equal to tissue diagnosis).

In our study definitively positive biliary brush cytology is defined as class 3 or higher.

Biliary duct stricture location was classified according to one thirds of total biliary duct length and was analyzed *via* two methods (apriori decided), once combining middle and lower common duct and once dividing the duct system to three equal parts. Other factors evaluated include length of stricture, mass size in CT scan/ultrasound, stricture dilation before brushing, age, and gender.

Statistical analysis

Descriptive statistical analysis with the calculation of means and standard deviations of the entire brushings cohort was carried out. Demographic variables were determined a priori as important factors in the outcome of the brushings. For categorical variables, a reference value was selected with the calculated odds ratio relative to the selected reference. Once significant variables were identified by univariate logistic regression analysis, a multivariate logistic regression was carried out. Sensitivity and specificity were calculated based on the gold standard of either surgical resection or clinical follow-up. All statistical analyses were carried out using Stata 8.0 (College station, TX).

RESULTS

Patient characteristics

A total of 199 patients met the inclusion criteria for the study. Ten patients with unknown final diagnoses were excluded. Findings from the remaining 189 patients were analyzed. The mean age was 66.3 (range 21-92) with 51.3% female. One hundred and twenty four patients (62.3%) had malignant and 65 (33%) benign strictures. Seventy seven (40.7%) of the biliary brushings were scored as class 3 or higher (i.e. malignant) and 112 (59.3%) as class 2 or lower (i.e. benign).

Cytological brushing confirmation was obtained in 62% (77/124) of patients with malignant strictures; however in the remaining 48 patients either FNA, surgical specimen or clinical course confirmed the malignant nature of the biliary stricture. Sixty-five patients had pancreatic adenocarcinoma, 10 had hepatic cancer, 16 had distal cholangiocarcinoma, 10 had presumed primary duodenal cancer, 4 had gallbladder cancer, and 3 had Klatskin tumor. The precise type of primary cancer was not identified in 16 patients, however, in all of them it was presumed to be pancreaticobiliary.

There was one non-neoplastic, false positive diagnosis. The patient was an 83-year-old man with a history of primary sclerosing cholangitis who developed a lower common duct stricture. Second brush cytology and ampullary biopsy however, revealed only reactive and inflammatory biliary epithelial changes. This patient was not subjected to surgery and is well 8 years later.

Sixty five patients had benign disease, most commonly strictures secondary to biliary stones (n = 38) and presumed sphincter spasm (n = 7). The rest had variety of benign diagnoses including primary sclerosing cholangitis (n = 4), chronic pancreatitis (n = 5), recurrent pyogenic cholangitis (n = 2), autoimmune liver disease, sarcoidosis, complex benign hilar stricture, leiomyoma, and anastomosis stricture.

Thirty six patients had repeat ERCP and brushing cytology from a period of one to ten months after initial negative cytology result. In 29 patients results of repeat cytology remained negative, however, seven patients were declared to have malignant stricture in repeat cytology. Adding the second cytology result to previous data led to an increase in sensitivity to 67% (83/124).

Overall, there were 76 true positive diagnoses, 64 true negative diagnoses, 48 false negative diagnoses, and one false positive diagnosis. The diagnostic sensitivity, specificity, PPV, and NPV of brush cytology for the series were 61%, 98%, 99%, and 57%, respectively.

Factors associated with positive yield

Among all variables studied including age, gender, location of stricture, CT scan findings, length of stricture, and endoscopic dilation prior to biliary brushing (Table 1), only age, mass size > 1 cm, and stricture longer than 1 cm are associated with positive cytology brushings. Variables associated with positive cytology brushing are illustrated in Table 2.

DISCUSSION

The examination of brush cytology is an established diagnostic technique in the investigation of patients with biliary strictures with suspected pancreatic, bile duct, ampullary, and gallbladder cancers. Acquisition of malignant cells by endoscopic brush cytology during initial ERCP is valuable in planning appropriate therapy and may be important for avoiding additional invasive procedures

Table 1 All variables studied for positive cytology brush

Variable	Value
Age (yr, range)	66.3 (21-92)
Gender (female)	51.30%
Location of stricture	
Lower CD	124
Mid + Upper CD	63
No stricture	12
CT scan findings	
CT not done	35
Mass not seen	95
Mass < 1 cm	5
Mass 1-3 cm	39
Mass > 3 cm	25
Length of stricture	
No defined stricture	14
Stricture < 1 cm	30
Stricture 1-3 cm	125
Stricture > 3 cm	30
Endoscopic dilation prior to brushing	15%
Final diagnosis	
Benign	65
Malignant	124
Unknown	10

CD: Common duct.

Table 2 Variables associated with positive cytology brushing

Variable	Odds ratio (95% CI)
Age	1.02 (1.00-1.05)
CT scan findings	
Mass < 1 cm	1
Mass 1-3 cm	2.22 (1.01-4.89)
Mass > 3 cm	2.86 (1.07-7.64)
Length of stricture	
Stricture < 1 cm	1
Stricture 1-3 cm	3.49 (1.18-10.2)
Stricture > 3 cm	7.70 (2.15-27.5)

for tissue diagnosis. We have reviewed brush samples from 199 patients during a time period of almost 4 years for yield, sensitivity, and specificity. This is one of the largest series of biliary brush cytology specimens reported and the largest and broadest study looking at factors associated with positive brush result.

The diagnostic sensitivity of biliary brushing has been relatively modest in most studies to date. Stewart and colleagues reviewed 406 patients and found the overall sensitivity to be 59.8%[13]. Similarly, Kurzawinski and colleagues[7,17] reviewed six early series and found the mean sensitivity to be 59%. However, two other large studies on brush cytology have recorded sensitivities of 35%-48% in the patients with pancreatico-biliary malignancy[5,8].

There are very few studies evaluating the factors influencing positive results. McGuire and colleagues have documented the association between the yield of enteropancreatic brush cytology and the location of malignancy, such that strictures of the head and body yielded higher rates of positive cytology compared to the strictures of ampulla, pancreatic genu, and tail regions[9]. DeBellis and colleagues have shown that stricture dilation does not improve the sensitivity of brush cytology for the detection of cancer. However, repeated brushing appears to increase the diagnostic yield[1].

We found that brush cytology accurately identified 76 of 124 (61.2%) neoplasms in our series, a similar result to those of earlier studies reviewed by Stewart and colleagues[13], Kurzawinski and colleagues[7,17], and by Foutch *et al*[3,4,18]. When we consider a second brushing in some patients our sensitivity increases to almost 70%. This is among the highest sensitivity reported in the literature. Other studies such as Lee *et al*[19,20], Ponchon *et al*[21], Longo *et al*[8,22], and Macken *et al*[23] all had lower sensitivities at 37%, 35%, 48%, and 57%, respectively. The reasons for the varied sensitivity are unclear.

Previous studies have shown that a diagnosis of carcinoma on brush cytology is highly specific. The specificity of biliary brush cytology in previous studies has been reported to be between 90%[17] to 100%[3,16,18,24-28]. There was one false positive diagnosis in our study (specificity 98%) in a patient with primary sclerosing cholangitis. Stewart and colleagues also reported three false positives cytological diagnoses among one hundred and sixty patients (specificity 98.1%)[13]. Sturm and colleagues also reported specificity of 97.2% with two false positive cytological diagnoses among 74 patients with benign strictures[30].

It has been suggested in previous studies that the limited sensitivity of brush cytology may be due to an inadequate cellular yield. Theoretically, stricture manipulation may increase exfoliation of tumor cells, making these cells more available for histopathologic evaluation, but DeBellis and colleagues have shown that stricture dilation does not improve the sensitivity of brush cytology for detection of cancer. In their study, brush cytology had a sensitivity of 34.5% before dilation and 31% after dilation ($P = 0.54$)[1]. On the other hand, Mohandas *et al*[31] found that dilation before bile aspiration improved the cancer detection rate of bile cytology. In their series, cytology was positive in 27% of cases without biliary stricture dilation compared to 63% when the stricture was dilated ($P < 0.03$). Use of scraping brush in the series reviewed by Parasher and Huibregtse[16] achieved a sensitivity of 100%. However, stricture dilation did not improve the diagnostic yield of brush cytology specimen in our series of 31 patients who had dilation prior to brush cytology OR 1.18 (0.48-2.85).

We have not routinely performed biliary aspiration or biopsy technique biopsies of the biliary tree; both of which may enhance diagnostic yield. Using a combination of simple biliary brushings occasionally with a second brushing combined with endoscopic ultrasound staging (and biopsy where required) sensitivity reaches more than 90%. Only a small selected group remains undiagnosed through the above two methods, and thus the benefit of other techniques (i.e. aspiration, endoscopic biopsy at ERCP) in increasing the yield is marginal in our hands.

We also analyzed positive yield for multiple factors including age, gender, location of stricture, mass size in CT scan or ultrasound, and the length of stricture. From all the mentioned variables positive brush result was associated with older age: 1.02 (1.00-1.05), mass size of more than 1 cm: 2.22 (1.01-4.89) and stricture longer than 1 cm: 3.49 (1.18-10.2).

In summary, in this study brush cytology identified 76 of 124 (61.2%) of neoplasms in a series of 199 consecutive patients evaluated during a 3.7 years period in our institution. One false positive cytological diagnosis occurred. From all the variables studied only age, length of stricture, and mass size in CT scan or ultrasound were associated with a higher yield. Gender, dilation of stricture prior to cytological sampling, or location of stricture were not found to be associated with a higher yield. Although the limitations of this technique must be recognized,, brush cytology is useful in investigation of patients with suspected biliary neoplasm. The challenge for future developments in this field is to develop strategies to improve lesion targeting facilitating retrieval of cytology specimens with higher quality.

COMMENTS

Background

The usage of biliary brushings has high specificity and moderate sensitivity in the diagnosis of malignant biliary obstruction. Although other methods of diagnosing the etiology of the obstructive lesion have become available we have evaluated our biliary tract brushing results to determine the sensitivity, specificity, and factors that might be associated with a positive result.

Research frontiers

Percutaneous biopsies have the risk of 'needle-track' seeding and thus are discouraged in patients deemed to be surgical candidates. Tissue from biliary/pancreatic lesions is also obtained through endoscopic ultrasound. We have not routinely used biliary aspiration or bile duct biopsies; both of which have a yield that may not be as high as reported in our study with biliary tract brushings.

Innovations and breakthroughs

We found that initial brush cytology accurately identified 76 of 124 (61.2%) patients with malignant biliary strictures. When a second brushing was performed in several patients, we obtained a sensitivity of close to 70%. This is much higher than most studies have reported. Theoretically, stricture manipulation may increase exfoliation of tumor cells, making these cells more available for histopathologic evaluation. Stricture dilation did not increase the yield. We also analyzed positive yield for multiple factors including age, gender, location of stricture, mass size in CT scan or ultrasound, and the length of stricture. From all the mentioned variables positive brush result was associated with older age: 1.02 (1.00-1.05), mass size of more than 1 cm: 2.22 (1.01-4.89), and stricture longer than 1 cm: 3.49 (1.18-10.2).

Applications

In this study, without the use of EUS, we still obtained a sensitivity of malignancy for biliary tract strictures of almost 70%. In this study we used Class 3 as a 'cut-off' for malignancy, however, some studies have used even more stringent criteria. When one then 'adds in' additional cytobiological results obtained with availability of EUS then the need for other methods to diagnose these lesions is limited to a very small group of patients.

Terminology

Biliary brushing: the use of a cytology brush through an endoscope to obtain samples for analysis. Biliary dilation: the use of either a step dilator or a balloon dilator to increase the size of the biliary tree lumen and to allow further intervention or to improve biliary flow.

Peer review

This is an important optimistic report on the diagnostic value of brush cytology in biliary strictures. The diagnostic yield is higher than it was reported in other previous studies. One additional aspect is the evaluation of factors influencing the results.

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RAPID COMMUNICATION

Gene modulation associated with inhibition of liver regeneration in hepatitis B virus X transgenic mice

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Abstract

AIM: To analyze the modulation of gene expression profile associated with inhibition of liver regeneration in hepatitis B X (HBx)-expressing transgenic mice.

METHODS: Microarray technology was performed on liver tissue obtained from 4 control (LacZ) and 4 transgenic mice (HBx-LacZ), 48 h after partial hepatectomy. The significance of the normalized log-ratios was assessed for each gene, using robust *t*-tests under an empirical Bayes approach. Microarray hybridization data was verified on selected genes by quantitative PCR.

RESULTS: The comparison of gene expression patterns showed a consistent modulation of the expression of 26 genes, most of which are implicated in liver regeneration. Up-regulated genes included DNA repair proteins (Rad-52, MSH6) and transmembrane proteins (syndecan 4, tetraspanin), while down-regulated genes were connected to the regulation of transcription (histone deacetylase, Zfp90, MyoD1) and were involved in the cholesterol metabolic pathway and isoprenoid biosynthesis (farnesyl diphosphate synthase, Cyp7b1, geranylgeranyl diphosphate synthase, SAA3).

CONCLUSION: Our results provide a novel insight into the biological activities of HBx, implicated in the inhibition of liver regeneration.

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Key words: Hepatitis B virus; Hepatitis B X (HBx) protein; Liver regeneration; Microarray analysis; Cholesterol; Isoprenoid

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INTRODUCTION

The liver has a unique capacity to regenerate after partial hepatectomy (PH) or injury[1]. Experimental evidence has confirmed that hepatocyte proliferation is responsible for liver regeneration following PH, despite the low replication rate of hepatocytes in a normal liver[1]. After a two-third partial hepatectomy, hepatocytes switch from a quiescent state to a proliferative state and re-enter cell cycle division to compensate for the loss of liver mass. Liver regeneration is a multi-step process comprising at least two critical points: the immediate early gene phase (priming) that mediates the transition of the quiescent hepatocytes to the cell cycle (G1), and progression from G1 to S phase. In mice, DNA synthesis peaks at 40-44 h after PH[1].

Numerous growth factors and cytokines regulate the regeneration process by providing stimulatory and inhibitory signals for proliferation. Tumour necrosis factor alpha (TNF-α) and interleukin-6 (Il-6), together with their downstream transcription factors Stat-3 and nuclear factor kappa B (NF-κB), are the most important initiating factors in the regenerative response[1]. TNF-

α-induced hepatocyte proliferation has been shown to involve the small GTPases (Ras and RhoA) necessary for cell cycle progression by stimulating the degradation of Cdk inhibitors ($p21^{Cip}$ and $p27^{Kip1}$)[2]. The initiation factors governing liver regeneration partially overlap with regulators of the hepatic acute phase response (APR), which results in dramatic changes in the expression of acute phase response proteins following PH[3]. Hepatocyte growth factor (HGF) and transforming growth factor alpha (TGFα) further stimulate cell cycle progression, DNA synthesis and cell proliferation. Finally, TGF-β and activin suppress cell growth and terminate liver regeneration at a fixed point[1]. Recently, a large scale gene expression analysis, based on cDNA microarray technology, has provided a unique opportunity to elucidate the expression pattern throughout the course of liver regeneration[4-6], and the priming phase of liver regeneration in mice[7].

In a previous report, we showed that hepatitis B virus (HBV) X protein (HBx) inhibits liver regeneration after PH in HBx-expressing transgenic mice, and also demonstrated a paracrine inhibitory effect of HBx on liver cell proliferation[8]. HBx exhibits pleiotropic effects that modulate cell responses to genotoxic stress, protein degradation, cell viability and signalling pathways[9]. HBx has been shown to enhance transcription in the cytoplasm by activating various signal transduction cascades such as Ras/Raf mitogen-activated protein kinase and Src kinases, as well as in the nucleus through transcription factors like AP-1, AP-2 and NF-κB. Several studies have identified possible cellular targets of HBx, including members of the cyclic-AMP response element binding protein (CREB)/activating transcription factor (ATF) family, the TATA-binding protein, RNA polymerase subunit RPB5, the UV-damaged DNA-binding protein and the replicative senescence $p55^{sen}$[9,10]. HBx has been shown to interact with p53 and inhibit its function, but it may also induce apoptosis *via* p53-dependent and independent mechanisms[9]. Furthermore, it has been demonstrated that mutations identified in HBx-encoding sequences in the tumour cells of HCC can markedly modify the biological activity of HBx, and possibly favour cell transformation[11]. It has been shown that the HBx protein participates in the development of hepatocellular carcinoma (HCC) in HBV infected patients[12]. Indeed, HBx protein can induce HCC in certain transgenic mice and may, under certain conditions, cooperate *in vitro* with Myc or Ras oncogene[13]. However, the mechanisms involved in HBx-mediated HCC remain obscure.

In the present study, we analyzed further the impact of HBx on gene expression profile in the liver of transgenic mice, following PH. For this purpose, we employed HBx-expressing transgenic mice mentioned above, and adopted a transcriptome approach to analyse gene expression modulation, 48 h after PH. We observed that impaired DNA synthesis and reduction in liver mass in HBx-transgenic mice was associated with a weaker expression of genes involved in the cholesterol and nonsterol isoprenoid pathways as well as in impaired expression of serum amyloid A.

MATERIALS AND METHODS

Source of RNA

Two groups of mice (the same as described previously[8]) were used as liver RNA source. HNF1-LacZ transgenic mice carrying the nls-LacZ gene driven by the hepatocyte nuclear factor (HNF-1) promoter acted as controls. Test animals AX16/HNF1-LacZ double transgenic mice, were generated by crossing homozygous AX16 transgenic mice carrying ORF HBx linked to the promoter/enhancer of human anti-thrombin Ⅲ gene, with HNF1-LacZ transgenic mice. PH was performed on 21-35 d old LacZ and HBx-LacZ (with confirmed expression of HBx) transgenic mice. Frozen liver tissue obtained from 4 control (LacZ) and 4 treated (HBx-LacZ) mice was pulverized in liquid nitrogen, 48 h after partial hepatectomy. Total RNA extraction was performed using RNAble (Eurobio), according to the manufacturer's instructions and purified by RNeasy kit (Qiagen) followed by on-column DNase digestion (RNase-free DNase set). Only RNAs with an absorbance ratio of 260/280 over 1.9 and intact ribosomal 28S and 18S rRNA bands, evaluated by 1% agarose gel electrophoresis, were utilized for the microarray study.

Microarray assay

The aminoallyl indirect labelling method was used to obtain Cyanin-3 (Cy-3) or Cy-5 labelled cDNA. Briefly, 20 μg of total RNA and 5 μg of oligo-dT primers were incubated at 70℃ for 10 min and snap-cooled in ice, in order to generate aminoallyl-labelled cDNA. Reverse transcription was then performed by adding 0.5 mmol/L dATP, dCTP, dGTP, 0.3 mmol/L dTTP, 0.2 mmol/L aminoallyl-dUTP (aa-dUTP), 400 U SuperScript Ⅱ, 10 mmol/L DTT, and 1 × first strand buffer. This mixture was incubated at 42℃. After RNA hydrolysis (1 U of RNaseH at 37℃ for 15 min), unincorporated aa-dUTP was removed by the QIAquick PCR purification kit (Qiagen). The resulting aminoallyl -cDNAs were diluted in 0.1 mol/L sodium carbonate (pH 9.0) and coupled with 100 μg of Cy-3 or Cy-5 monoreactive dye (Amersham), prepared in dimethyl sulfoxide (DMSO) for 1 h. Cy3 and Cy5 labelled cDNA targets were concentrated in a Microcon filter device (Millipore) and suspended in 15 μL of hybridization buffer (0.7% SDS, 70% formamide in 3.6 × Denhardt solution). The cDNA microarray consisting of 5376 mice and rat cDNA spotted onto glass slides (Functional Genomic Service, CEA) was prehybridized in buffer containing: 3.5 × sodium chloride-sodium citrate buffer (SSC), 1% Bovine Serum Albumin (BSA) and 0.1% sodium dodecyl sulfate (SDS) for 1 h at 50℃. For each experiment, test cDNA was mixed with control cDNA, labelled with Cy3. Mixed, labelled cDNA, after preheating to 42℃ in hybridization buffer, was overlaid on dried glass cDNA array and incubated overnight at 42℃. After hybridization, the slides were washed once in 2 × SSC/0.2% SDS buffer and twice in 0.2 × SSC buffer, and dried by centrifugation.

Microarray analysis

For each slide, the fluorescent images of hybridized microarray were scanned with a Gene Pix 4000B scanner.

Table 1 Primer's sequence and PCR conditions used for the microarray results validation

Name of gene	Sequences of forward (F) and reverse (R) primers	Source of primers	PCR product (bp)	T anneling (℃)	Mg^{2+} (mmol/L)
Serum amyloid A3	F: TCAGCACATTGGGATGTTTAGG R: CAGAGGACTCAAGAGCTGACCA	UniSTS: 219434	206	53	3
Creatine kinase muscle	F: CCTCCTGGAAGTCCAATCAT R: GGCCATCACGGACTTTTATT	UniSTS: 159603	150	53	3
Peroxiredoxin 1	F: GAGCAGCCAGAAGAAACTCTTG R: AGAAGATTGGTCTGCCCAAAA	UniSTS: 144118	153	53	3
Retinoblastoma-like 2	F: TGGCTGAGTCCTGTAACAAC R: CCAACACCTTTCTGAGGC	UniSTS: 162222	374	53	2
Interleukin-6-receptor, 80-kD	F: AAGCAGCAGGCAATGTTACC R: CATAAATAGTTCCCAGTGTCG	[18]	120	55	3
DNA mismatch repair protein MSH6	F: ATATGTCCTAGGCGCACACAAA R: CTAGCATACTCAGGCATGCGAC	UniSTS: 211063	208	56	4
Peroxisome proliferator-activated receptor-α	F: CATCGAGTGTCGAATATGTGG R: GCAGTACTGGCATTTGTTCC	[19]	172	55	4
β-actin	F: CGTGACATTAAGGAGAAGCTGTGC R: CTCAGGAGGAGCAATGATCTTGAT	[20]	374	53	2

MSH6: DNA mismatch repair protein 6.

Image analysis was performed using a Gene Pix Pro 40054 to quantify the arrays. Spots flagged automatically by Genepix or by visual inspection of the array scans were excluded from the analysis. Global normalization was applied to correct the artefacts caused by different incorporation rates or scanner settings for two dyes. Scatter plots in a log scale were performed to visualize fold changes between two channels by plotting Cy5 intensity against Cy3 intensity. Normalization steps were performed using Bioconductor array packages[14]. After removal of the background, we normalized the log2 of the cy5/cy3 ratio using a print-tip loess approach[15]. For each gene, the average of the normalized log-ratios was computed and the significance was assessed using robust *t*-tests under an empirical Bayes approach[16] as implemented in the limma package. *P*-values were then corrected by the FDR approach[17] to take account of test multiplicity. Differentially expressed genes were considered to be those with a false discovery rate (FDR) of less than 5% and an expression ratio > 1.4.

Real-time PCR

In order to verify microarray hybridization data, real-Time PCR was performed using a Light Cycler rapid thermal system (Roche Diagnostics). Random cDNA was transcribed from 10 μg total RNA with 400 U of Superscript Ⅱ in the presence of 1 μg random primers, according to the manufacturer's instructions. All PCR experiments were carried out on the same cDNA preparation. Reactions were performed according to the manufacturer's instructions in a 20 μL volume containing 2 μL of 10-fold diluted cDNA, 0.5 μmol/L of primers, and an $MgCl_2$ concentration optimized between 2 and 4 mmol/L. A typical protocol included 10 min of initial denaturation followed by 40 cycles of 95℃ denaturation for 20 s, annealing at 53℃-56℃ for 15 s and a 72℃ extension for 5-12 s (depending on the PCR product). For each set of primers, a template without reverse transcription was amplified as a negative control. The details of primer sequences and amplification conditions are summarized in Table 1. The quantification of gene expression was based on a standard curve prepared from gene-specific, purified PCR product (PCR clean-up kit, Macherey-Nagel). The specificity of each PCR product was controlled by a melting curve analysis and subsequent agarose gel electrophoresis of the PCR product. For each experiment, relative concentrations were obtained after normalization with β-actin values. Calculation of the ratio of each mRNA expression was based on the relative concentration of specific cDNA found by RT-PCR in samples originating from control and HBx-expressing livers.

RESULTS

Microarray analysis of gene expression in the liver of HBx-transgenic mice after partial hepatectomy

As previously reported, HBx expression inhibits liver regeneration after PH in AX16/HNF1-LacZ transgenic mice, by a combination of intracellular and paracrine effects[8]. In the present study, we investigated, using microarray analysis consisting of 5376 murine genes, the impact of HBx expression on gene expression profile, 48 h after partial hepatectomy to analyze any changes in gene expression which may be involved in the observed inhibition of liver regeneration in HBx transgenic mice. This time point of gene expression analysis was chosen in order to determine alterations in gene expression associated with the DNA synthesis phase[1]. In addition, BrdU incorporation analysis performed on SCID mice transplanted with HBx-expressing hepatocytes, 48 h after partial hepatectomy, had indicated a marked (5.5-fold) reduction in the cellular DNA synthesis[8].

RNAs isolated from the livers of four HBx-LacZ mice were labelled separately with Cy5, and RNAs from the livers of four LacZ mice were mixed together and labelled with Cy3 as the reference. Thus, the expression profile of each of the four tested RNA was compared with the expression of control RNA by the microarray assay (performed in duplicate). 82% of the clones spotted

Table 2 Upregulated genes in HBx-LacZ transgenic mice 48 h after partial hepatectomy

Gene name (Abbreviation)	Unigene ID	GB Acc.	Fold difference HBx-LacZ/LacZ	*P* value	Function
Aven: caspase activation inhibitor	Mm.292041	BF662037	1.50	0.030	Caspase inhibitor
RAD52 homolog	Mm.149	U12135	2.14	0.035	DNA repair/double-strand break
Mismatch repair protein MSH6	Rn.16755	XM345633	1.51	0.009	DNA repair/mismatch
Epidermal growth factor	Rn.6075	NM012842	1.40	0.014	Growth factor
DC-SING (CD209)	Mm.32510	NM133238	1.40	0.009	Transmembrane protein/Cell adhesion
Leucyl-tRNA synthetase	Hs.432674	NM020117	1.41	0.009	Metabolism/protein synthesis
Syndecan 4	Mm.3815	BC005679	1.42	0.014	Transmembrane protein/Focal adhesion
Transmembrane 4 superfamily member	Mm.18590	BC050153	1.45	0.009	Tetraspanin interacting with beta-1 integrin
Golgi SNAP receptor complex member 2	Rn.13518	BC061994	1.42	0.011	Transmembrane protein/Vesicular transport of Golgi

Abbreviations: RAD52: DNA double-strand break repair and recombination protein; DC-SING: Dendritic Cells-specific intercellular adhesion molecule 3-grabbing nonintegrin.

Table 3 Downregulated genes in HBx-LacZ transgenic mice 48 h after partial hepatectomy

Gene name (Abbreviation)	Unigene ID	GB Acc.	Fold difference HBx-LacZ/LacZ	*P* value	Function
Serum amyloid A3 (Saa3)	Mm.14277	X03479	-1.95	0.016	Acute phase response protein cholesterol transport
Cadherin 16 (Cdh 16)	Mm.19423	AF016271	-2.64	0.010	cell recognition protein
Creatine kinase (Ckm)	Mm.2375	AI325205	-1.65	0.040	ATP synthesis
Glutathione S-transferase, pi2 (Gstp2)	Mm.299292	AI325120	-1.85	0.043	Detoxification and drug metabolism
Progastricsin (pepsinogen C) (Pgc)	Mm.22957	AK008959	-1.73	0.009	Digestion enzyme
Phospholipase A2 group 1B (Pla2g1b)	Mm.20190	AI327450	-1.58	0.016	Digestion enzyme
Tripsin Ⅱ precursor (Try2)	Mm.301947	AI386046	-1.54	0.014	Digestion enzyme
Farnesyl diphosphate synthase (Fdps)	Mm.39472	W76783	-1.98	0.020	Enzyme of cholesterol pathway
Cytochrom P450, 7b1 (Cyp7b1)	Mm.278588	U36993	-1.61	0.009	Enzyme of cholesterol pathway
Geranylgeranyl diphosphate synthase 1 (Ggps 1)	Mm.148039	AB016044	-1.41	0.034	Enzyme of cholesterol pathway
Aldolase1, A isoform (Aldo 1)	Mm.275831	AI327494	-3.35	0.015	Enzyme of glycolysis pathway
Peptidylglycine alpha-amidating monooxygenase	Mm.5121	AI323455	-1.73	0.029	Posttranslational modification
Mouse integrase gene (IN)	NF	X52622	-1.43	0.021	Replication
Myogenic differentiation1 (MyoD1)	Mm.1526	M84918	-1.52	0.032	Transcription
Zinc finger protein 90 (Zfp 90)	Mm.295582	X79828	-2.06	0.040	Transcription
Histone deacetylase 10 (Hdac 10)	Mm.346413	AI323 456	-3.73	0.014	Transcription
Prion protein (Prnp)	Mm.648	M13685	-1.40	0.021	CNS protein

on the microarray revealed hybridization signals, and the data shown in Tables 2 and 3 was obtained after the elimination of genes that did not comply with the criteria for expression, normalization and statistical analysis (see Material and Methods). In order to validate the microarray analysis, real-time PCR was performed on selected genes for which the expression was found to be up- or down-regulated or unmodified in LacZ-HBx mice when compared with LacZ mice. As shown in Table 4, the RT-PCR results were similar to the data obtained from cDNA microarrays; we therefore did not perform RT-PCR on all the genes listed in Tables 2 and 3.

As shown in Table 2, the majority of the up-regulated genes in HBx-expressed transgenic mice corresponded to the expression of DNA repair and transmembrane proteins. RAD52 is responsible for DNA double-strand break repair and mitotic recombination and MSH6 for DNA mismatch repair[21]. The transmembrane protein genes implicated in focal adhesion (syndecan 4), viral internalization (CD209), transport of protein between the medial-and trans-Golgi compartment (Golgi SNAP receptor) and in interactions with beta-1 integrin (transmembrane 4 superfamily member 2) were found to be up-regulated. In addition, changes in the expression of the epidermal growth factor and Aven, a caspase inhibitor, were also observed.

Genes whose expression was found to be down-regulated in the liver of HBx-expressed transgenic mice (Table 3) consisted of two main classes: those connected to the transcription control, such as Myogenic differentiation1, Zinc finger protein 90 and Histone deacetylase 10 (HDAC 10), and genes involved in cell metabolism, such as serum amyloid A3 (SAA3), creatine kinase, pepsinogen C, aldolase A, glutathione S-transferase and phospholipase A2. A subclass of down-regulated genes, connected with cholesterol metabolism and isoprenoid synthesis, such as farnesyl diphosphate synthase (FPPS), geranylgeranyl diphosphate synthase (GGPPS) and Cyp7 were also observed. In the group of genes that was found to be down-regulated in the liver of HBx-transgenic mice, we observed several genes like SAA3, aldolase A, creatine kinase, phospholipase A2 that have been reported elsewhere as up-regulated during the course of liver regeneration[22-24].

Interestingly, down-regulation of histone deacetylase and up-regulation of DNA repair genes (Rad52 and MSH6)

Table 4 Results from RT-real time PCR 48 h after PH for selected genes

Gene	Mice[1]	RT-real time PCR		Microarrays fold difference HBx-LacZ/LacZ
		Relative value[2]	Fold difference HBx-LacZ/LacZ	
SAA3	LacZ	0.37 ± 0.41	-10.9	-2.00
	HBx-LacZ	0.03 ± 0.02		
Creatine Kinase	LacZ	1.36 ± 1.29	-1.6	-1.65
	HBx-LacZ	0.87 ± 0.91		
MSH6	LacZ	1.08 ± 0.64	1.5	1.50
	HBx-LacZ	1.62 ± 0.64		
Rb2	LacZ	0.47 ± 0.15	1.2	1.20
	HBx-LacZ	0.59 ± 0.23		
Peroxiredoxin	LacZ	0.08 ± 0.02	1.5	1.40
	HBx-LacZ	0.12 ± 0.08		

SAA3: Serum Amyloid A3; MSH6: DNA mismatch repair protein 6; Rb2: Retinoblastoma like-2. [1]Four animals in control (LacZ) group and four animals in treated (HBx-LacZ) group; [2]for each experiment relative values were obtained after normalization with beta actin expression.

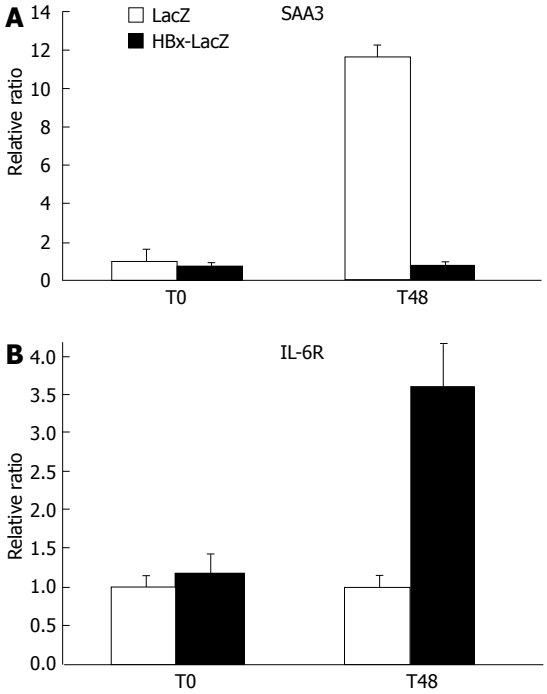


Figure 1 Expression levels of SAA3 (**A**) and IL-6R (**B**) transcripts in livers obtained from LacZ and HBx-LacZ transgenic mice. RNA was isolated before (T0) and 48 h after (T48) partial hepatectomy from the liver of two HBx-expressing (HBx-LacZ) and two control (LacZ) transgenic mice. The level of transcript expression was analyzed by quantitative RT-PCR and expressed as relative ratios after normalization with β actin values. The means of the relative ratio values and standard error were obtained from at least two independent experiments performed in duplicate.

observed in our study in HBx-transgenic mice after PH have, in agreement with other reports, similar effect on the cell cycle by inducing cell cycle arrest[25-28]. Furthermore, it has previously been reported that HBx expression may interfere with nucleotide excision repair mechanisms[29,30] and interact with the DNA repair protein[31,32].

In the next subsections we will focus on selected modulated genes implicated in cholesterol regulation and discuss their possible impact on the inhibition of liver regeneration.

Modulation of serum amyloid A expression and liver regeneration

SAA3 is a multifunctional protein involved in acute phase response, inflammation, and cholesterol transport. However, the precise function of SAA in liver regeneration is unclear. The physiological transient induction of SAA appears to be indispensable for the precise regeneration rate after PH[3]. To investigate whether HBx expression was able to completely or partially inhibit SAA3 expression during PH, real time RT-PCR was performed on liver biopsies obtained at the time of PH (T0) and 48 h later from two LacZ or HBx-LacZ transgenic mice. As expected, in the control LacZ animals, a marked increase in SAA expression (more than 10-fold) was observed 48 h after PH as compared to T0 (Figure 1A). By contrast, in HBx-LacZ mice, PH was not associated with an increase in the expression of SAA3 transcript. Thus, HBx induces complete inhibition of SAA response during PH. This may constitute an important factor involved in the impaired liver regeneration previously observed in these animals.

Regulation of SAA expression during the acute phase response depends on hepatic mitogens (Il-6, IL-1, TNF-α)[3]. As the expression of these cytokines is not significantly modified by HBx 48 h after PH, they are probably not implicated in the observed inhibition of SAA3 transcription in HBx-LacZ transgenic mice. To define whether any modulation of the expression of Il-6 receptor (IL-6R), not incorporated in our microarray, is related to the inhibition of SAA expression in HBx-LacZ mice, we used RT-PCR assay to determine the expression of IL-6R in LacZ and HBx-LacZ transgenic mice, at the time of PH and 48 h later. As shown in Figure 1B, prior to PH, no difference in IL-6R expression was observed in HBx-LacZ mice compared to control mice. By contrast, 48 h after PH there was greater than 3-fold up-regulation in HBx-LacZ mice as compared to controls. These findings suggest that HBx is able to modulate Il-6 receptor expression in association with a factor activated during PH and confirms that the lack of SAA response after PH in HBx-expressing transgenic mice is independent of cytokine activity.

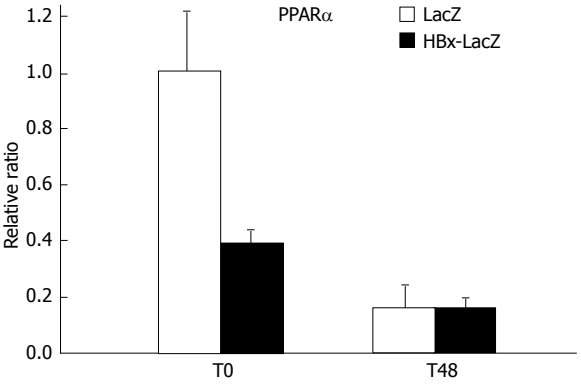


Figure 2 Expression levels of PPARα transcripts in the liver of two HBx-expressing (HBx-LacZ) and two control (LacZ) transgenic mice, before (T0) and 48 h after (T48) partial hepatectomy. The level of transcript expression was analyzed by quantitative RT-PCR and expressed as relative ratios after normalization with β actin values. The means of the relative ratio values and standard error were obtained from at least two independent experiments performed in duplicate.

Impaired expression of isoprenoid pathway and peroxisome proliferator-activated receptor-α

We observed that after PH of HBx-transgenic mice, the expression of enzymes implicated in the isoprenylation biosynthesis (FPPS and GPPS, and to a lesser extent HMG-CoA synthase) was reduced in the liver. Peroxisome proliferator-activated receptor-α (PPARα) is a transcription factor that controls lipid metabolism and glucose homeostasis. PPARα regulates the expression of enzymes implicated in isoprenoid synthesis, and its role in liver regeneration after PH was demonstrated via mechanisms involving prenylation of small GTPases Ras and RhoA independent of TNF-α and IL-6 activation[33-35]. In addition, it was observed that PPARα is involved in the expression of FPPS and HMG-CoA synthase after PH[33]. Thus, we wanted to investigate whether the decrease in the expression of enzymes implicated in isoprenylation observed in HBx-transgenic mice can be explained by a decrease in PPARα expression. We used RT-PCR to assess PPARα expression in LacZ and HBx-LacZ transgenic mice, both at the time of PH and 48 h later. At the time of PH, a 60% decrease in the expression of PPARα gene was observed in HBx-LacZ compared to LacZ transgenic mice (Figure 2). It has been reported that the absence of PPARα expression in mice contributes to impaired liver regeneration[36]. As previously reported, there is a general decrease in PPARα expression after PH[37]. In accordance with the microarray data, there was no difference in PPARα expression between LacZ and HBx-LacZ transgenic mice, 48 h post PH. Thus, the reduced expression of PPARα before PH may be involved in the inhibition of liver regeneration in HBx-LacZ transgenic mice.

DISCUSSION

In the present study, we used liver biopsies of HBx-expressing transgenic mice, 48 h after partial hepatectomy, to assess the factors implicated in the inhibition of liver regeneration in HBx-transgenic mice, by determining the alterations in mRNA profile. By that means, we extended the results obtained in *in vivo* studies, demonstrating inhibition of liver regeneration by HBx-expression in HBx-transgenic mice[8]. The present study demonstrates the influence of altered expression of genes involved in transcription control, DNA reparation, cholesterol metabolic pathway and isoprenoid biosynthesis in the reduction of liver mass observed in HBx-transgenic mice[8].

Although the data is controversial, it has been shown that HBx may up-regulate the expression of $p21^{waf1/cip1}$ and prolong G1→S transition[38,39]. A recent study on liver regeneration performed on transgenic mice showed that HBx can block G1/S transition, and so cause the lower liver mass restoration[40]. In this context, we showed that HBx down-regulated HDAC10 and up-regulated DNA repair gene (Rad52 and MSH6) expression, which may have a similar impact on the cell cycle. Indeed, HDAC inhibition causes cell cycle arrest due to an increase in the expression of p21 WAF1/Cip1 and Rb[25,26,41,42]. Moreover, it has been reported that Rad52 over-expression affects cell cycle regulation by delaying the exit from G1[27], and that MSH6 protein induces apoptosis by activation of p53[28]. Thus, one of the possible mechanisms that has emerged from our study is in accordance with previously reported activity of HBx, inhibition of cell cycle progression throughout the modulation of pathways implicated in DNA repair and transcription control.

During regeneration induced by tissue loss, a complex of growth factors and cytokines induce hepatocyte progression from G0 to cell cycle to restore the original liver mass. At the same time, Il-6, IL-1 and TNF-α, known to be hepatic mitogens, induce the acute-phase response (APR)[3,43]. During APR, the hepatic biosynthesis of SAA is up-regulated by proinflammatory cytokines, and the circulating concentration of SAA can increase up to 1000 fold. SAA is induced transiently and returns to its normal low basal level within 72-96 h of the initial stimulus[44]. It has been reported earlier that the mRNA for APR protein such as serum amyloid A increases dramatically during liver regeneration[45]. When we analyzed the changes in the mRNA profile in liver 48 h after PH we also observed a greater than 10-fold increase in the level of SAA mRNA, but only in the control mice. SAA expression level in HBx-transgenic mice 48 h after PH remained unchanged (even slightly lower) when compared to T0. Although the exact function of SAA is still unclear, the physiologic transient induction of SAA appears to be indispensable for proper regeneration after PH. Thus, the lack of SAA response in HBx-transgenic mice after PH may constitute an alternative factor in the impaired regeneration.

The activity of HMG-CoA synthase, farnesyl pyrophosphate synthase and geranylgeranyl pyrophosphate synthase was found to be indispensable for the isoprenylation process and the subsequent cell cycle progression and cell proliferation[33,34]. Furthermore, FPPS and GGPPS are implicated in cell cycle progression via their association with the isoprenylation process of GTPases Ras and RhoA proteins[33,34,46]. In addition, PPARα plays an important role in regulating lipid homeostasis. Studies of PPARα null mice have revealed the importance of PPARα in the hepatic lipid metabolism and liver regeneration[33,36]. Based on our cDNA microarray

results we determined that after PH of HBx-transgenic mice the expression level of these enzymes in the liver is down-regulated. As shown in previous studies, inhibition of enzymes involved in cholesterol and isoprenoid synthesis leads to the upregulation of cdk inhibitors, reduction in cdk, hypophosphorylation of Rb, followed by cell cycle arrest in G1[47,48]. Thus, the tempered liver regeneration in HBx-transgenic mice can be explained by the inhibition of the isoprenylation process.

Taken together, our microarray experiments performed on HBx-transgenic mice have provided new mechanisms for HBx-mediated inhibition of liver regeneration. The relevance of our results in the context of HBV-dependent cell cycle inhibition, required for an efficient HBV replication, awaits further evaluation.

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COMMENTS

Background

The liver has a unique capacity to regenerate after partial hepatectomy (PH) or injury. Experimental evidence supports the hypothesis that proliferation of hepatocytes is responsible for liver regeneration after PH, despite the low replicative rate of hepatocytes in the normal liver. In a previous report we had shown that HBx expression inhibits liver regeneration after PH in HBx expressing transgenic mice. We also demonstrated a paracrine inhibitory effect of HBx on liver cell proliferation. Indeed, transplantation of HBx-expressing liver cells or injection of serum from HBx-transgenic mice into control mice is sufficient to inhibit liver regeneration after PH. Thus, HBx protein may act by combining the intracellular and paracrine effects, and inhibiting the proliferation of both infected and uninfected liver cells in the context of liver regeneration.

Research frontiers

Hepatitis B Virus (HBV) infection is a worldwide health problem. It is estimated that 350 million people are chronic carriers of hepatitis B virus (HBV). According to the WHO, hepatitis B is responsible for 1.2 million deaths each year. Chronic HBV infection is a major risk factor for the development of hepatocellular carcinoma. The HBx protein of HBV exhibits pleiotropic effects, that modulate transcription, cell responses to genotoxic stress, protein degradation, cell viability and signalling pathways. Several studies have suggested that HBx protein of HBV, participates in the development of HCC, although the precise role(s) in this process remain unclear.

Innovations and breakthroughs

In the present report, we examined the impact of HBx on the gene expression profile of liver cells in HBx-expressing transgenic mice, 48 h after partial hepatectomy. We used cDNA microarray containing over 5 thousand mouse and rat cDNA elements to determine genes that are altered in the presence of reduced liver regeneration. By demonstrating molecular mechanisms involved in HBx-mediated inhibition of liver regeneration, the data provides a better understanding of the role of the HBx protein in the physiopathology of chronic liver disease caused by HBV infection.

Applications

Although these observations were made in the specific context of liver regeneration triggered by partial hepatectomy, they may represent an important mechanism of HBV-dependent liver carcinogenesis in chronic hepatitis and the associated stimulation of liver cell proliferation. Thus, this study may contribute to the development of new approaches based on controlling the cellular effects of viral replication and ultimately provide new tools to control HBV-induced liver disease.

Peer review

This is a well written, interesting report on gene modulation associated with inhibition of liver regeneration in hepatitis B x protein transgenic mice.

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RAPID COMMUNICATION

Gli-1 siRNA induced apoptosis in Huh7 cells

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Abstract

AIM: To investigate the effects of Gli-1 small interference RNA (siRNA) on Huh7 cells, and the change of Bcl-2 expression in Huh7 cells.

METHODS: Human hepatocellular carcinoma cells Huh7 were used. Cell viability was analyzed by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The expressions of Gli-1 and Bcl-2 family members were detected by RT-PCR and Western blot. Apoptosis was detected by Flow cytometry using propidium iodide, measured by Hoechst 33258 staining using Advanced Fluorescence Microscopy and caspase-3 enzymatic assay. Cell growth was analyzed after treatment with Gli-1 siRNA and 5-fluorouracil (5-Fu).

RESULTS: Inhibition of Gli-1 mRNA in Huh7 cells through Gli-1 siRNA reduced cell viability. Gli-1 siRNA treatment also induced apoptosis by three criteria, increase in the sub-G1 cell cycle fraction, nuclear condensation, a morphologic change typical of apoptosis, and activation of caspase-3. Gli-1 siRNA was also able to down-regulate Bcl-2. However, Gli-1 siRNA resulted in no significant changes in Bcl-xl, Bax, Bad, and Bid. Furthermore, Gli-1 siRNA increased the cytotoxic effect of 5-Fu on Huh7 cell.

CONCLUSION: Down-regulation of Bcl-2 plays an important role in apoptosis induced by Gli-1 siRNA in HCC cells. Combination Gli-1 siRNA with chemotherapeutic drug could represent a more promising strategy against HCC. The effects of the strategies need further investigation *in vivo* and may have potential clinical application.

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Key words: Gli-1 transcription factor; Small interference RNA; Apoptosis; Hepatocellular carcinoma; 5-fluorouracil

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INTRODUCTION

Hedgehog (Hh) gene was first identified in a *Drosophila* genetic screen for mutations affecting body patterning[1]. This pathway contains Hh proteins (Sonic Hh, Indian Hh, and Desert Hh), the twelve transmembrane protein Patched (Ptc), the seven transmembrane protein Smoothened (Smo), and the five-zinc finger transcription factor Glis (Gli-1, Gli-2, and Gli-3)[2-4]. When Hh binds to Ptc, Smo is released and the downstream transcription factors such as Gli-1 are activated. Conversely, when Hh unbinds to Ptc, Ptc prevents Smo from activating the pathway[5]. Glis proteins are regarded as nuclear executors of hedgehog signaling[6,7]. Evidence from several groups suggests that Gli-1 and Gli-2 represent the main activators of Hh-target genes, while Gli3 acts mainly as repressor[8,9]. Gli-1 has been demonstrated to represent a direct transcriptional Hh target gene and the marker of Hh pathway activation[10-13].

Constitutive activation of the Hh pathway has been shown to contribute to the growth and maintenance of various cancers[14-21]. We have previously shown that Hh pathway activation was an important event for the development of human hepatocellular carcinoma (HCC)[22]. Our results corroborated and extended the findings of Sicklick *et al*[23] about disregulation of Hh signaling in human hepatocarcinogenesis. The fact that the Hh pathway is active in human HCC suggests a significance of novel targeted strategy for HCC therapy.

Sustained application of specific Hh-pathway inhibitors has been proven effective in preventing growth of many tumors with activating Hh signaling *in vitro* and *in vivo*[21-26], suggesting a promising approach of Hh signaling interference. Current strategies focus on the use of small

molecule inhibitors of Smo. Blocking Smo with antagonist, e.g. cyclopamine or KAAD-cyclopamine[27] is a reasonable yet limited approach, since it is only effective for tumors with Hh pathway activation occurring upstream or at the level of Smo. However, tumors with inactivating mutations in Smo or downstream components will most likely not respond to Smo antagonists[25,28]. It has been reported that Gli-1 is the last and essential step of the pathway and plays a central role in HCC proliferation[24]. Thus, blocking Gli-1 transcription factor would be an ideal strategy to combat HCC. In this paper, we investigated the effects of Gli-1 small interfering RNA (siRNA) on the human liver cancer cells. Our results indicated that Gli-1 siRNA induced apoptosis through down-regulation of Bcl-2 in HCC cells.

MATERIALS AND METHODS

Reagents

The primary goat polyclonal anti-Gli-1 and nonspecific control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit monoclonal anti-Bcl-2, SABC kit, and horseradish peroxidase-conjugated mouse anti-goat/rabbit IgG secondary antibody were obtained from Boster (Wuhan, China). 5-fluorouracil (5-Fu), TRIzol reagent, and propidium iodide (PI) were obtained from Sigma Chemical Co (St. Louis, MO, USA). 5-Fu was dissolved in phosphate buffered solution (PBS).

Cell culture

The human HCC cell line Huh7 and human hepatocyte cell line L02 were kindly donated by professor Guan Xin-yuan (Hong Kong University). Huh7 and L02 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% (*v*/*v*) fetal bovine serum (Bio-Whittaker, Walkersville, MD, USA) at 37℃ in a humidified atmosphere with 5% CO_2.

Immunocytochemistry assay

Immunocytochemical staining was performed as described by the manufacturer on cell slides by the streptavidin-biotin-peroxidase complex method using a SABC kit. For negative control, the primary anti-Gli-1 antibody was replaced with PBS.

Treatment with siRNA

The Gli-1 and Bcl-2 siRNA reagent kits were purchased from RiboBio Co. LTD (Guangzhou, China). siRNAs were transfected using the LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions after cells were grown to 50% confluence. Briefly, 1 μL LipofectAMINE was diluted in 50 μL OptiMEMI Reduced Serum Medium (Invitrogen, Carlsbad, CA, USA), mixed gently, and incubated for 5 min at room temperature. Subsequently, a mixture of siRNA was added and incubated for 20 min. The mixture was diluted with medium and the final concentration of siRNA was 100 nmol/L. After treatment for the indicated time period, cells were collected for further investigation.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for cell viability

Cells were seeded onto 96-well plates at 5000 cells/well overnight. After incubating with the test compounds for the indicated time period, 20 μL MTT (5 g/L MTT in PBS) was added to each well and incubated at 37℃ for 4 h. The cells were then centrifuged (700 × *g* for 15 min), and the supernatant was removed. DMSO (200 μL/well) was added to dissolve the cell pellets. Absorbance was measured at a wavelength of 570 nm with enzyme-linked immunity implement (BIO-RAD 2550, USA).

Flow cytometry assay

Huh7 cells were incubated with each experimental treatment. Cells were collected, washed twice with pre-cold PBS, and fixed with 70% ethanol overnight at 4℃. Cell pellets were resuspended in PBS after being washed twice with pre-cold PBS. Then 50 μL of RNase (10 μg/mL) and 25 μL of propidium iodide (1 mg/mL) were added to the cells for 30 min at room temperature, and then flow cytometry (EPICS-XL, Beckman Coulter, Fullerton, USA) analysis was performed. Data were analyzed using CellQuest Software (Becton Dickinson, Mountain View, CA, USA).

Chromatin staining with Hoechst 33258

Apoptosis was observed by chromatin staining with Hoechst 33258 as previously described[29]. In brief, after 48 h transfection with Gli-1 siRNA, Huh7 cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, washed again with PBS, and stained 10 min with Hoechst 33258 (5 mg/L), washed, and observed under an Advanced Fluorescence Microscope (NIKON 80i, Tokyo, Japan) by an observer blind to the cell treatment.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from Huh7 cells with TRIzol reagent. First strand cDNA was synthesized using total RNA with the ReverTra Ace system (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. Transcripts of the gene for β-actin were used as an internal control. We used the following primers for PCR: Gli-1 (sense: 5'-ttcctaccagagtcccaagt-3', antisense: 5'-ccctatgtgaagccctattt-3', 185 bp), Shh (sense: 5'-CGCA CGGGGACAGCTCGGAAGT-3', antisense: 5'-CTGC GCGGCCCTCGTAGTGC-3', 476 bp), Ptch (sense: 5' -GTTGGAAGAAAACAAACAGC-3', antisense: 5'-AG CCGTCAGGTAGATGTAGA-3', 277 bp), Smo (sense: 5'-TTACCTTCAGCTGCCACTTCTACG-3', antisense: 5'-GCCTTGGCAATCATCTTGCTCTTC-3', 321 bp); Bcl-2 (sense: 5'-CTGGTGGACAACATCGC-3', antisense: 5'-GGAGAAATCAAACAGAGGC-3', 135 bp), Bcl-xl (sense: 5'-GTAAACTGGGGTCGCATTGT-3', antisense: 5'-TGGATCCAAGGCTCTAGGTG-3', 146 bp), Bax (sense: 5'-CCAGCTGCCTTGGACTGT-3', antisense: 5'-ACCCCCTCAAGACCACTCTT-3', 135 bp), Bad (sense: 5'-AGGGCTGACCCAGATTCC-3', antisense: 5'-GTGACGCAACGGTTAAACCT-3', 178 bp), Bid (sense: 5'-GCTTCCAGTGTAGACGGAGC-3', antisense:

5'-GTGCAGATTCATGTGTGGATG-3', 116 bp)[30] and β-actin (sense: 5'-GTGGACATCCGCAAAGAC-3', antisense: 5'-GAAAGGGTGTAACGCAACT-3', 303 bp). The reactions were carried out in a DNA Thermal Cycler (Perkin-Elmer Corp. Norwalk, Connecticut, USA) with the following cycle conditions: denaturation at 94℃ for 1 min, annealing at respective temperature for 30 s, and extension at 72℃ for 1 min, for 35 cycles.

Western blot analysis

Western blotting was performed as described previously[31,32] with minor modification. Total cell extracts were prepared by lysing cells in RIPA lysis buffer. For gel electrophoresis, equivalent of total protein (40 μg) was loaded on each lane of a 10% sodium dodecyl sulfate-polyacrylamide gel.

Caspase-3 activity detection

After treatment, activation of caspase-3 was detected with 200 μg of cell lysate using the colorimetric protease assay kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer's protocol. The activity of caspase-3 was converted through measurement of absorbance (OD 405 values) using a microplate reader at 405 nm. Non-specific reactions were corrected by subtracting background absorbance readings from the combination of cell lysate and buffer.

Statistical analysis

Values were expressed as mean ± SD from at least three separate experiments. Differences between groups were assessed with one-way ANOVA or Student's *t*-test using SPSS 11.5 for Windows (SPSS Inc, Chicago, IL, USA). Differences were considered significant if $P < 0.05$.

RESULTS

Expression of Hh pathway components in Huh7 cells

To begin to elucidate the effect of Gli-1 on Huh7 cells, we first examined the expressions of Hh pathway components in Huh7 cells and the normal hepatocyte cell line L02 cells. The expressions of Hh pathway components were determined by RT-PCR analyses. The expressions of Gli-1 were also assayed by immunocytochemistry detection. RT-PCR analyses showed that Shh, Smo and Gli-1 were expressed in Huh7 cells, but not in L02 cells (Figure 1A). Ptch was detected in both cells, however, the expressions were elevated in Huh7 cells. To further confirm these findings, we performed immunocytochemistry for Gli-1. Gli-1 protein was present in the cytoplasm of Huh7 cells, but not in L02 cells (Figure 1B). Because Gli-1 is one of the indicators of the Hh pathway activation, our data indicated that Hh pathway is active in Huh7 cells.

Gli-1 siRNA reduced Huh7 cell viability

Since Hh pathway is activated in Huh7 cells, we investigated whether Gli-1 siRNA could kill Huh7 cells. Firstly, we characterized the function of Gli-1 siRNA on Gli-1 mRNA expression. Huh7 cells were transfected with Gli-1 siRNA or nonspecific control siRNA at a concentration of 100 nmol/L. Cells treated with PBS were used as a mock control. Twenty-four hours and forty-eight hours after treatment, the expressions of Gli-1 mRNA were assayed by RT-PCR. The results indicated that Gli-1 siRNA completely blocked Gli-1 expressions in Huh7 cells at two time points (Figure 2A and B). Subsequently, the cell viability was assayed by MTT. The results showed that Gli-1 siRNA decreased cell viability to 88.3% ± 2.3% and 76.8% ± 3.4% after twenty-four hours and forty-eight hours treatment, respectively (Figure 2C).

Gli-1 siRNA induced apoptosis in Huh7 cells

To test whether cell viability decreases by Gli-1 siRNA through apoptosis, we evaluated morphologic and biochemical changes typical of apoptosis. As illustrated in Figure 3A, chromatin condensation was seen by Hoechst 33258 staining forty-eight hours after Huh7 cells treated with 100 nmol/L of Gli-1 siRNA. As apoptosis progresses into the late stage, apoptotic bodies with partially degraded DNA and DNA cleaved at internucleosomal sites appear. These apoptotic bodies were detected as the sub-G1 fraction on propidium iodide fluorescent flow cytometry analysis. Huh7 cells were treated for forty-eight hours with Gli-1 siRNA and subsequently stained with propidium iodide for flow cytometry. The cellular DNA content analysis revealed that Gli-1 siRNA induced more apoptosis than did either control or negative control (Figure 3B). The average percentages of the sub-G1 cells are plotted in Figure 3C. Gli-1 siRNA significantly increased the percentages of the sub-G1 cells by 15.8% ± 2.6%. Caspase-3 is classically regarded as executor. The caspase-3 activities in cell lysates were assayed using a colorimetric assay kit. In accordance with the increase of sub-G1 cells, Gli-1 siRNA increased 2.01-fold caspase-3 activity compared with the activity in untreated control cells (Figure 3D). These results indicated that Gli-1 siRNA reduced Huh7 cell viability through apoptosis induction.

Gli-1 siRNA mediated apoptosis by decreased expressions of the Bcl-2 gene

Several cytoplasmic proteins are critically involved in the regulation of apoptosis, in particular, members of the Bcl-2 family[33-35]. Gli-1 has been shown to up-regulate expressions of the key anti-apoptotic factor Bcl-2 in some malignancies[36,37]. To determine whether Gli-1 siRNA-induced apoptosis in Huh7 cells was associated with change of Bcl-2 family, Bcl-2 family was analyzed after treatment with Gli-1 siRNA. The expressions of Bcl-2 mRNA were remarkably decreased twenty-four hours after treatment with Gli-1 siRNA. In contrast, Bcl-xl, Bax, Bad, and Bid were unchanged (Figure 4A). Consequently, we used western blot to detect Bcl-2 protein. Treatment with Gli-1 siRNA for twenty-four hours resulted in an obvious decrease of Bcl-2 expression. To investigate whether Gli-1 is upstream of Bcl-2, Huh7 cells were treated with Bcl-2 siRNA. The findings showed that the expressions of Gli-1 were unchanged (Figure 4C). These results together with the data presented above indicated that Gli-1 was upstream of Bcl-2 in the hierarchical pathway of apoptosis regulation.

Gli-1 siRNA increased cytotoxic effects of 5-Fu in Huh7 cells

Hedgehog cascades have been proved to be involved in

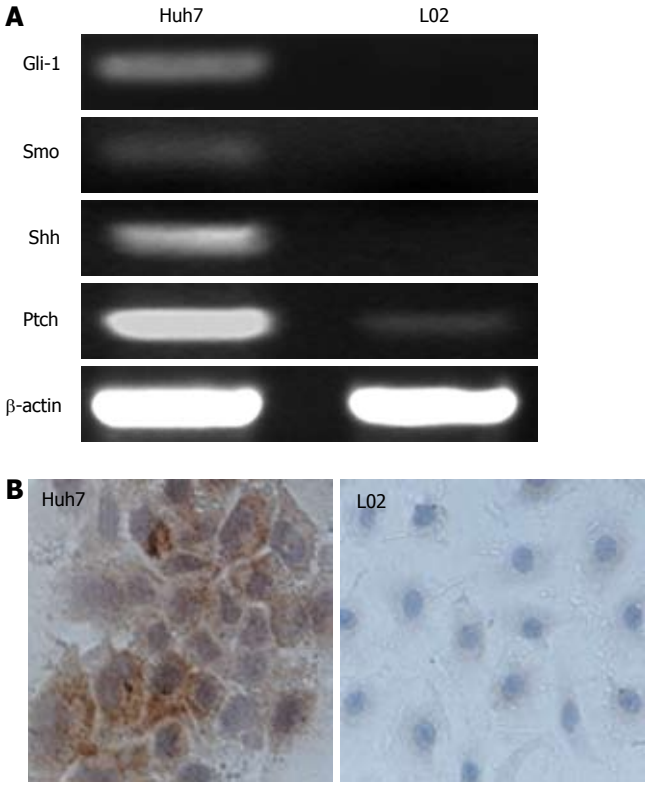


Figure 1 Expressions of Hh pathway components on Huh7 and L02 cells. **A**: The expressions of Gli-1, Smo and Shh mRNA was observed in Huh cells, but not in L02 cells; Ptch mRNA was detected in both cell lines, but the expressions was elevated in Huh7 cells; **B**: Immunocytochemical staining showed Gli-1 protein expression in the cytoplasm of Huh7 cells, but not L02 cells.

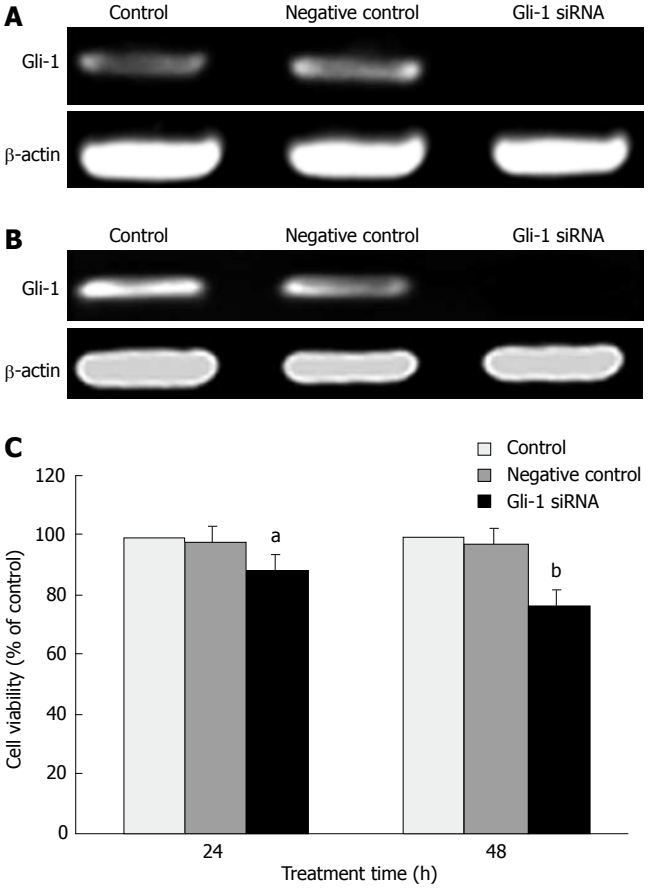


Figure 2 The effect of Gli-1 siRNA on cell viability in Huh7 cells. **A**: The expressions of Gli-1 mRNA 24 h after transfection with Gli-1 siRNA analyzed by RT-PCR. Gli-1 mRNA expressions were significantly down-regulated; **B**: The expressions of Gli-1 mRNA 48 h after transfection with Gli-1 siRNA analyzed by RT-PCR; **C**: The effect of Gli-1 siRNA on Huh7 cell viability by MTT assay. Cells were treated with Gli-1 siRNA for indicated time. Data are expressed as the mean ± SD of 3 independent experiments ($^aP < 0.05$, $^bP < 0.01$ *vs* control).

the resistance to clinical therapies and disease relapse in some cancers[38-40]. We further tested if Gli-1 siRNA could increase the cytotoxic effects of 5-Fu. The results showed that significant increased cytotoxicity effects were observed in Huh7 cells treated with Gli-1 siRNA plus 5-Fu (Figure 5). Moreover, Gli-1 siRNA enhanced 5-Fu-induced apoptosis in Huh7 cells (Table 1).

DISCUSSION

Hedgehog signaling pathway has been found to play a critical role in regulating the growth, proliferation, and apoptosis of several cancers. Various types of cancers contain Hh activation. We have shown previously that hedgehog signaling is activated in HCC[22]. In this study, we found that Hh pathway components including Gli-1, the marker of Hh pathway activation, were expressed in the HCC cell line Huh7 but not in L02 normal liver cells. This provides a target to preferentially kill HCC cells.

Previous studies demonstrated that the naturally occurring plant extract cyclopamine or KAAD-cyclopamine that directly associates with the transmembrane domains of SMO could inhibit proliferation, induce apoptosis in a subset of HCC cell lines[22,24]. These observations suggest a possibility for developing new therapeutic strategies using the Hh pathway inhibitor to kill cancer cells with Hh pathway activation. The Hh pathway can be blocked at several steps, such as blocking Smo with antagonist, e.g. cyclopamine or KAAD-cyclopamine[22-24,41], Shh antibody[17,20] and Gli-1 RNA interference[25]. Blocking Smo with an antagonist can generally inhibit the growth of tumor cells with activated Hh signaling, however, this drug may be less effective in tumors with genetic mutations downstream of SMO[42]. Mutation of Smo was found in human HCC[24]. Effective treatment of Huh7 cells will require identification of novel small molecular weight compounds acting downstream of Smo signaling. Thus, we hypothesized that Gli-1 siRNA that directly inhibit Gli-1 could be effective for Huh7 cells. The present study investigated the effects of Gli-1 siRNA on Huh7 cells. We demonstrated that Gli-1 siRNA down-regulated expression of Gli-1 and significantly decreased cell viabilities in Huh7 cells *via* inducing apoptosis. These findings are consistent and extended with the findings of Chen *et al* regarding induction of apoptosis and decrease of cell proliferation in ovarian carcinoma cells[43] and of Huang *et al* regarding Gli-1 which may be an important regulator in survival of HCC cells[22]. Furthermore, these observations are in agreement with the mechanism of the Hh pathway blockage reported previously. Nevertheless, our findings in this paper are unlike that of Gli-2 in playing a dominant role over Gli-1 in regulating the proliferation of HCC cells[44]. Thus, even in the same cell

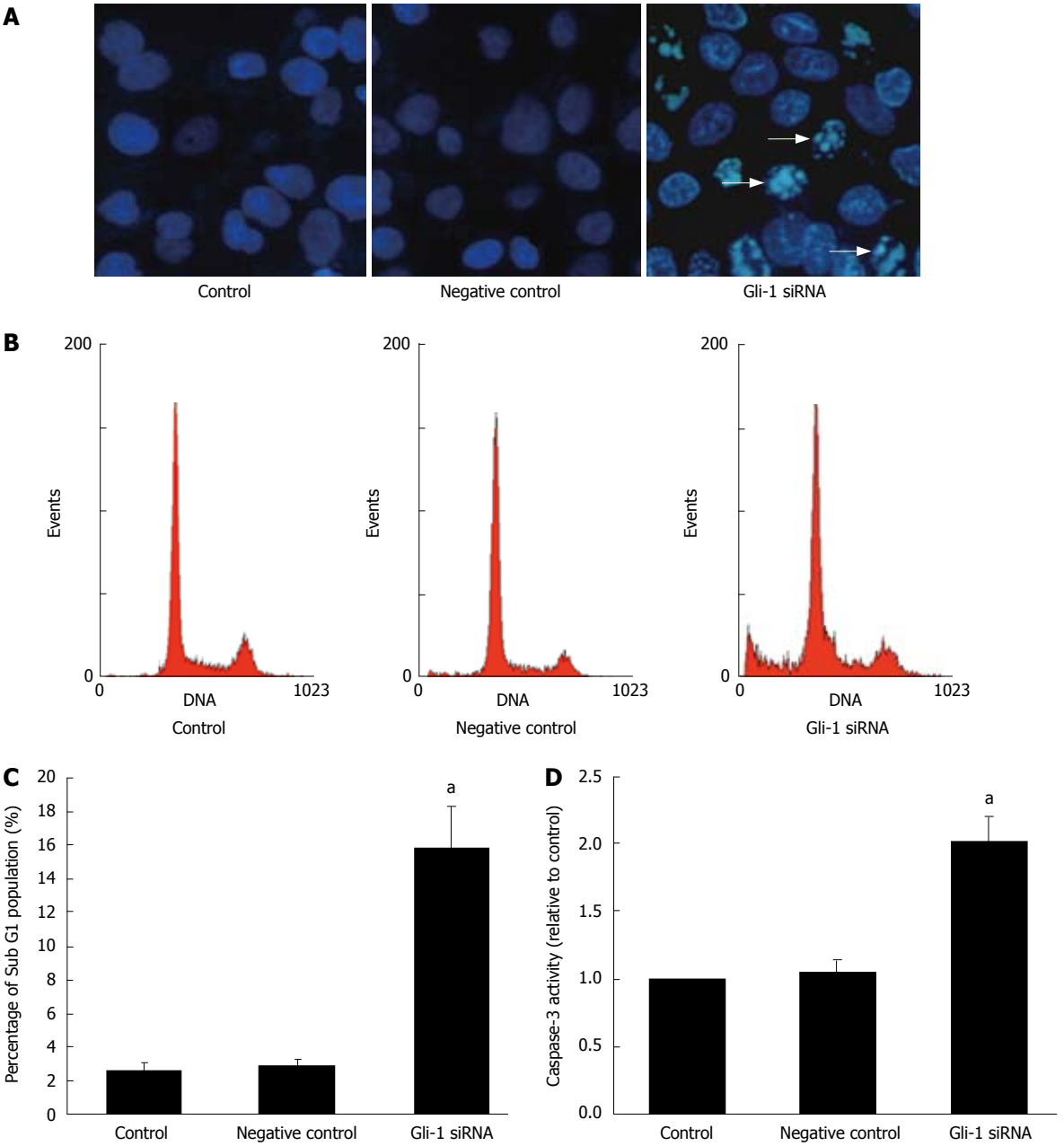


Figure 3 Gli-1 siRNA induced apoptosis in Huh7 cells. Huh7 cells were treated with Gli-1 siRNA for 48 h. **A**: Morphologic changes characteristic of apoptosis. The samples were stained with Hoechst 33 258 (5 mg/L) for 10 min and photographed using fluorescence microscopy; **B**: Gli-1 SiRNA induced apoptosis as measured by propidium iodide using a flow cytometry analysis. After treatment, the nuclei were stained with propidium iodide and analyzed by flow cytometry. Representative frequency distributions of DNA content of cells in the three treatment groups were shown; **C**: The bar chart showed the percentage of cells in the sub-G1 apoptotic fraction. The treatment groups were labeled on the horizontal axis. The error bars represented the standard deviation; **D**: Gli-1 siRNA induced activation of caspase-3. The activity of caspase-3 was assessed by caspase colorimetric assay. The data were showed as mean ± SD of 3 independent experiments. ($^aP < 0.05$ *vs* control).

line, it seems that inhibition of Gli-1 has different effects on cell survival according to the type of knock down method. However, it is still possible that inhibition of Gli-2 may have more cytotoxic effect on HCC cells. It will be worthwhile to further evaluate. On the other hand, the cytotoxic effects of siRNA were moderate. This may be because of autocrine Shh expression in Huh7 cells that continuously drive the expression of Gli-1 to lower the effect of siRNA. Our unpublished data indicate that in low concentration of Gli-1 siRNA-treatment, Gli-1 almost recovers after twenty-four hours of treatment even when Gli-1 is silenced completely at the twelve hour time point. The findings suggest that autocrine Shh have impact on the effect of Gli-1 siRNA. It should be given the rational concentration to exert the best effect of Gli-1 siRNA on HCC.

The Bcl-2 family proteins are important regulators of apoptosis. Among the members of this family, Bcl-2 and Bcl-xl act as inhibitors of apoptosis, whereas Bax and Bak act as promoters of apoptosis. Mitochondrial apoptotic pathway is activated while Bax is over-expressed and transported to the mitochondria. Conversely, Bcl-2 inhibits Bax activity through binding to Bax. Thus, Bcl-2 was thought to be a very important anti-apoptotic protein in cells[34,35]. Several studies showed the increased expression of Bcl-2 in HCC contributed to cell death inhibition[45,46].

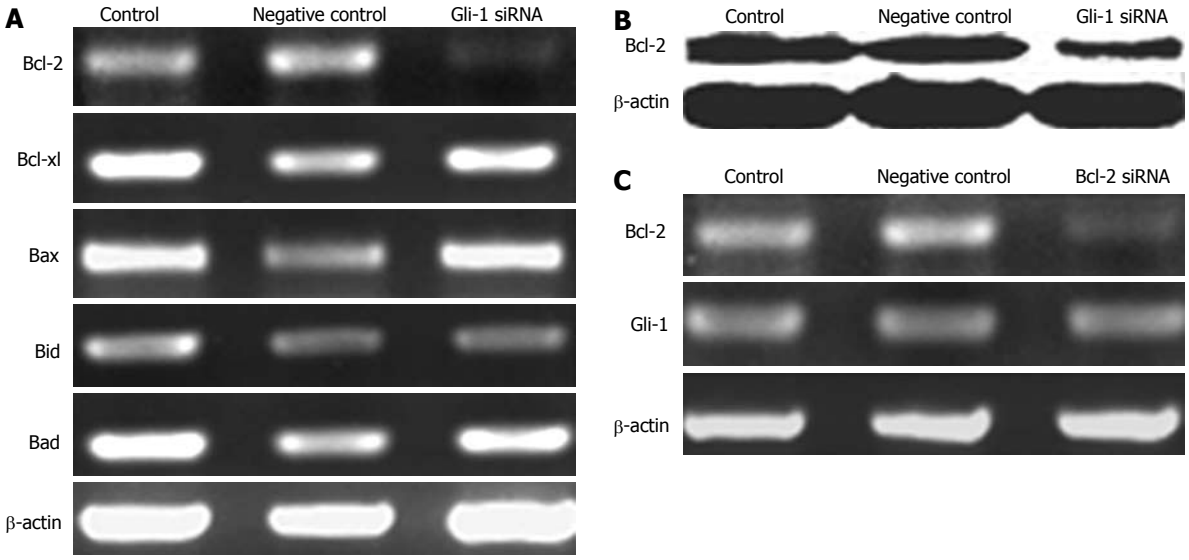


Figure 4 Effects of Gli-1 siRNA on Bcl-2 family member expressions in Huh7 cells. **A**: The expressions of Bcl-2 family member mRNA 24 h after transfection with Gli-1 siRNA analyzed by RT-PCR. The expression of Bcl-2 was remarkably decreased, but no change for other members; **B**: The protein of Bcl-2 was detected by Western blot. The Bcl-2 protein was markedly decreased after 24 h treatment with Gli-1 siRNA; **C**: Bcl-2 siRNA effectively inhibited the expressions of Bcl-2, but the mRNA of Gli-1 was no change after transfection with Bcl-2 siRNA.

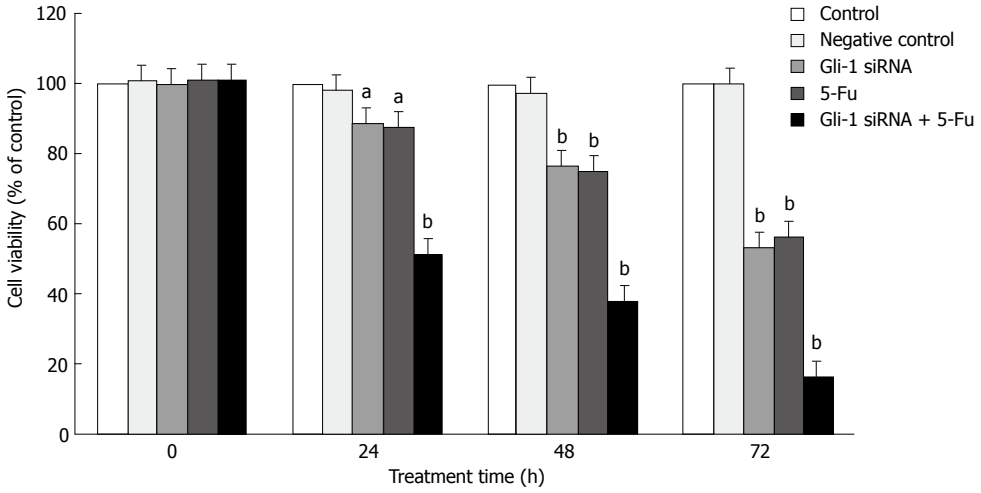


Figure 5 Effect of Gli-1 siRNA combining with 5-Fu on Huh7 cells. The effect of Gli-1 siRNA combining with 5-Fu on Huh7 cell viability was measured by MTT assay. Cells were transfected with Gli-1 siRNA. After 6 h, cells were exposed to 5-Fu (25 μmol/L) or not for the indicated time periods. The bars are grouped according to the treatment time as labeled below the horizontal axis. The treatment groups were as labeled on the color key. Data are expressed as the mean ± SD of 3 independent experiments ($^aP < 0.05$; $^bP < 0.01$ *vs* control or negative control).

It is of interest to establish a direct link between Hh/Gli signaling and Bcl-2 expression. Bigelow *et al* showed that Gli1 was able to stimulate the Bcl-2 promotor[37]. Morton *et al* recently also demonstrated that activation of hedgehog signaling protected tumor cells from apoptosis through the stabilization of Bcl-2 protein[47]. We showed that Gli-1 siRNA induced apoptosis in Huh7 cells. On the basis of the findings reported here it is reasonable to assume that the effect of Gli-1 siRNA would, at least in part, be mediated by the regulation of Bcl-2 expression. In the present study, we showed that Gli-1 siRNA remarkably reduced the level of Bcl-2 mRNA expressions and Bcl-2 protein, but was not effective on other Bcl-2 family members. Moreover, the level of Gli-1 mRNA had no change after transfection with Bcl-2 siRNA in Huh7 cells. These findings suggest that over-expression of Bcl-2 is a consequence of the activation of Gli-1. Thus, silencing Gli-1 would induce apoptosis through down-regulation of Bcl-2 expressions in Huh7 cells. Nevertheless, the involved apoptotic pathway of Gli-1 siRNA-induced apoptosis in Huh7 cells through down-regulation of Bcl-2 remains to be investigated.

5-Fu is the most common chemotherapeutic agent in advanced HCC treatment. However, drug-resistance results in failure of chemotherapy. Loss of apoptosis pathway promoted by Hh signaling activation has been suggested to be the key mechanism by which HCC is resistant to 5-Fu. On the basis of the ability of Gli-1 siRNA to induce apoptosis in Huh7 cells, one would expect that Gli-1 siRNA should increase sensitivity of Huh7 cells to 5-Fu. In fact, the present study showed that Gli-1 siRNA increased chemosensitivity significantly. These results suggest that it is indeed possible to combine Gli-1 siRNA and 5-Fu to overcome drug resistance and enhance therapeutic activity. It would be important to further test the effect of other combinations of Gli-1 down-regulating agents with 5-Fu on a variety of HCC cells. Its potential therapeutic implications seem to justify future research efforts in this area.

In summary, our data provide a potential mechanism

Table 1 Combined effect of Gli-1 siRNA and 5-Fu on apoptosis in Huh7 cells

Group	Percentage of sub G1 population (%)
Control	2.63 ± 0.28
Negative control	2.78 ± 0.39
Gli-1 siRNA	15.8 ± 2.13[a]
5-Fu	27.29 ± 3.14[a]
5-Fu + Gli-1 siRNA	44.25 ± 4.59[a,c]

Huh7 Cells were transfected with Gli-1 siRNA. After 6 h, cells were treated in the presence or absence of 25 μmol/L 5-Fu for 48 h. After treatment, the nuclei were stained with propidium iodide and analyzed by flow cytometry. The percentage of cells in the sub-G1 apoptotic fraction is shown in table. The data shown as mean ± SD were from three independent experiments. ANOVA analysis showed difference between groups was significant ([a]$P < 0.05$ *vs* control or negative control; [c]$P < 0.05$ *vs* other treatment groups).

for Gli-1 siRNA-induced apoptosis. Our findings suggest that combination Gli-1 siRNA with chemotherapeutic drug could represent a more promising strategy against HCC. The effects of the strategies need further investigation in other HCC cell lines and *in vivo*.

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COMMENTS

Background

Hedgehog pathway plays an important role in the development and maintenance of human hepatocellular carcinoma (HCC). Thus, there has been increasing interest in the application of specific Hh pathway inhibitors in cancer therapy for many years. Current strategies focus on the use of small molecule inhibitors of Smo. However, tumors with inactivating mutations in Smo or downstream components will most likely not respond to Smo antagonists. Gli-1 is the last and essential step of the pathway and would be the ideal target for HCC therapy. However, the effects of Gli-1 small interfering RNA (siRNA) on the human liver cancer cells remains unclear.

Research frontiers

We demonstrated that Gli-1 siRNA significantly decreased cell viabilities *via* inducing apoptosis through down-regulation of Bcl-2 expression in Huh7 cells. Furthermore, Gli-1 siRNA increased cytotoxic effects of 5-Fu in Huh7 cells.

Innovations and breakthroughs

Our data provide a potential mechanism for Gli-1 siRNA-induced apoptosis. Moreover, our findings show that Gli-1 siRNA significantly increased sensitivity of chemotherapy and suggest that combination Gli-1 siRNA with a chemotherapeutic drug could represent a more promising strategy against HCC. Overcome drug-resistance *via* Hh pathway inhibition will be a big breakthrough in HCC therapy. To our knowledge, this is the first discovery of Gli-1 siRNA causing apoptosis *via* down-regulation of bcl-2.

Applications

Our findings suggest that combination Gli-1 siRNA with chemotherapeutic drug could represent a more promising strategy against HCC. Furthermore, findings of Gli-1 siRNA significantly induced apoptosis through down-regulation of Bcl-2 expression are important in understanding regulation of apoptosis and a rational mechanism-based design of chemotherapy combinations for future clinical testing. Since about 75% of cancer cells of various origins and types are sensitive to drugs with a similar mechanism of action, the findings in this study are highly likely to be applicable to a wide range of cancers. Understanding these molecular mechanisms will allow us to identify novel therapeutic targets and design innovative combination chemotherapy regimens.

Terminology

Gli-1: It is a glioma-associated oncogene homologue 1 gene, Gli-1 is thought to be a direct transcriptional Hh target gene and the marker of Hh pathway activation. Bcl-2: is a family composed of various members, which are generated in mitochondria and key regulators of apoptosis. Bcl-2 is an anti-apoptotic protein and exhibits high expression in a wide range of human cancers including HCC. The increased expression of bcl-2 contributed to cell death inhibition. SiRNA: Small Interfering RNA (siRNA) is a 21-23-nt double-stranded RNA molecule. It guides the cleavage and degradation of its cognate RNA.

Peer review

This paper reports that Gli-1 siRNA induces apoptosis of human hepatocellular carcinoma cells and that this is mediated by down-regulation of Bcl-2. Furthermore, Gli-1 siRNA increased the cytotoxic effect of 5-Fu on Huh7 cells. These results extend the authors' previous observations and in agreement with the mechanism of Hh pathway blockage reported previously. The authors tried to identify novel therapeutic targets and design a more promising strategy based on Hh pathway inhibitor against HCC.

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RAPID COMMUNICATION

Alleviation of ischemia/reperfusion injury in ob/ob mice by inhibiting UCP-2 expression in fatty liver

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Abstract

AIM: To investigate the protective effect of target suppression of uncoupling protein-2 (UCP-2) on ischemia/reperfusion (I/R) injury in fatty liver in ob/ob mice.

METHODS: Plasmids suppressing UCP-2 expression were constructed, and transfected into fatty liver cells cultured *in vitro* and the ob/ob mouse I/R injury model. Serum tumor necrosis factor (TNF)-α levels, UCP-2 mRNA expression, alanine aminotransferase (ALT) levels in ob/ob mice were tested, and the pathological changes in fatty liver were observed in experimental and control groups.

RESULTS: In ob/ob mouse I/R models, serum TNF-α levels were significantly higher than in normal controls. After the plasmids were transfected into the cultured cells and animal models, expression of UCP-2 mRNA was significantly reduced as compared with that in the control group ($2^{1.56 \pm 0.15}$ *vs* $2^{-0.45 \pm 0.15}$, $P < 0.05$). In ob/ob mouse models, in which expression of UCP-2 was suppressed, serum ALT levels were significantly lower than those of other groups, and pathological analysis revealed that injury of liver tissues was significantly alleviated.

CONCLUSION: The target suppression of UCP-2 expression in fatty liver can alleviate the I/R injury in the ob/ob mice.

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Key words: ob/ob mice; Fatty liver; Uncoupling protein-2; Ischemia/reperfusion injury

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INTRODUCTION

It has been reported that primary graft non-function (PNF) occurs in over 25% of donor liver grafts with a lipid volume of > 30%[1,2]. Previous studies have discovered that PNF of fatty liver after liver transplantation is related to liver ischemia/reperfusion (I/R) injury during transplantation[3-5]. Recently, it has been discovered that one kind of mitochondrial protein, uncoupling protein-2 (UCP-2), may play an important role in I/R injury of liver transplantation[6,7]. UCP is a protein of the inner mitochondrial membrane and acts as a proton carrier to allow the dissipation of the proton electrochemical gradient, without phosphorylation of ADP to ATP, which results in the uncoupling of oxidation and ATP synthesis[8-10]. The increased expression of UCP-2 leads to a decrease in ATP in liver mitochondria, therefore, the fatty liver is more susceptible to injury than the normal liver under stress, e.g. transplantation[11]. Previous studies have indicated that polyunsaturated fatty acids can stimulate UCP-2 expression in fatty hepatocytes *via* a peroxisome proliferation activated receptor-α (PPAR-α) mediated pathway[12,13], and reactive oxygen species (ROS) generated intracellularly during lipid metabolism participate in UCP-2 induction[14,15], In addition, tumor necrosis factor (TNF)-α also increases mitochondrial oxidant production and induces expression of UCP-2 in fatty hepatocytes[16,17]. In the previous study by Wan *et al*, caerulomycin (a specific inhibitor of fatty acid synthetase) preconditioning suppressed the expression of UCP-2 in the fatty liver of ob/ob mice, which suggests that preconditioning alleviates the injury of liver cells following transplantation[18]. Caerulomycin cannot be widely used in clinical practice because it leads to metabolic disorders[19,20]. In this study, an antisense technique was used to directly suppress the expression of UCP-2 in fatty liver tissues, and the protective effects of UCP-2 expression inhibition on I/R injury in ob/ob mice was evaluated.

MATERIALS AND METHODS

Materials

Female 4-6-wk-old obesity mice (C57BL ob/ob) and inbred 10-12-wk-old normal mice (C57BL lean) were bought from the Model Animal Research Center of Nanjing University (China). Other reagents and equipment were from the following sources: collagenase Ⅳ (Gibco, USA), Percoll (Second Factory of Shanghai Biochemical Reagents, China), HEPES (Sigma, USA), EGTA (Sigma, USA), Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA), TNF-α (Sigma, USA), fetal bovine serum (FBS, Shanghai Huamei Bio-engineering Co., China), Ca^{2+}-free Hank's solution and PBS (self prepared), ELISA kit (R&D, USA), TRIzol (Gibco, USA), SYBR Green I fluorescent probe, FTC-200 type real-time fluorescent quantitative PCR instrument and fluorescent quantitative PCR analysis software (Shanghai Fengling Bio-tech, China), CO_2 incubator (Heraeus, USA), UCP-2 primer (Shanghai Bio-engineering), RibocloneR M-MLV(H-) cDNA synthesis system (Promega, USA), eukaryotic expression carrier pEGFP2-C2 (Clontech, USA), restriction endonucleases *Xho* I and *Sal* I , T4 DNA lygase (Huamei Bio-engineering Co), Taq enzyme (Takara, Japan), plasmid extraction purification kit, PCR recycling kit and gel DNA recovery kit (Shanghua Shunhua Bio-engineering, China).

Experimental animals and groupings

There were five *in vitro* experimental groups: normal hepatocyte culture (lean liver cells, A1 group); normal hepatocyte culture + TNF-α (lean liver cells + TNF-α, A2 group); fatty liver cell culture (A3 group); fatty liver cell culture + TNF-α treatment (A4 group); and fatty liver cell culture + TNF-α treatment + UCP-2 inhibition (A5 group). There were three *in vivo* experimental groups ($n = 20$ in each group): normal mouse I/R group (lean mice, B1 group); ob/ob mouse I/R group (obesity mice, B2 group); and ob/ob mouse I/R + UCP-2 inhibition (B3 group).

Mouse I/R model and primary culture of hepatocytes

Under anesthesia, mice were laparotomized, the portal and hepatic veins were ligated by non-invasive vessel clamp for 15 min, then re-opened to establish the I/R model. Hepatocytes were isolated using the collagenase perfusion method[21]. Mice were anesthetized and laparotomized to explore the liver hilus. After intubation of the portal vein, HEPES-buffered Hank's solution (pH 7.4) containing EGTA was perfused into the portal vein at 37℃, and subsequently, the tissues were perfused with collagenase Ⅳ buffer (0.5%) containing Ca^{2+} for digestion. Digested hepatocytes were harvested, re-suspended in culture medium, filtered through a sieve, centrifuged at low speed, and washed with PBS. Non-parenchymal cells were removed with Percoll by centrifugation. Hepatocytes were washed with PBS, centrifuged and stained with Trypan blue to assess cell viability. Hepatocytes were prepared into single cell suspensions and added to a six-well culture plate (1.2×10^6 cells/well) with addition of 2 mL DMEM containing 5% FBS incubated in a 5% CO_2 incubator at 37℃. Eight hours later, the solution was changed, and then changed every 48 h.

Construction and transfection of UCP-2 antisense plasmids

Mouse UCP-2 mRNA was extracted from adipose tissue and a cDNA library. Mouse full-length UCP-2 cDNA was synthesized by the Riboclone M-MLV(H-) cDNA synthesis technique and PCR. The primer was designed according to the Green fluorescent protein (GFP) reading frame of eukaryotic expression carrier pEGFP2-C2, which allowed the coding region reading frame of UCP-2 consistent with that of GFP. Antisense plasmid was constructed with a pair of primers with SalI restriction sites at the 5' end and XhoI restriction sites at the 3' end. Sequences of primers used for construction of UCP-2 fluorescent eukaryotic expression carrier, which was synthesized by Shanghai Bio-engineering was as follows:

P1 *Sal* I 5'-3': 5'-ATGTCGACGTCGGAGATACCAGAG CACT-3';

P2 *Xho* I 5'-3': 5'-TTCTCGAGGTGACCTGC GCTGTG GTACT-3'.

PCR products were cut by *Xho* I and *Sal* I restriction enzymes and linked to the enkaryotic expression carrier pEGFP-C2 (pEGFP-C2-UCP2-antisense). After the construction was identified to be successful, the transfected competent Escherichia coli were cultured and the extracted plasmids were recombined with reverse transcriptional viruses. In B3 group, the mice were transfected with UCP-2 plasmids via the portal vein at the same time as the vessel opening.

Sampling and detection

In all cultured cell groups, cells was added with different concentration of TNF-α (0, 2.5, 5, 10 ng/mL) at 48 h. In A5 group, fatty liver cells were transfected with UCP-2 plasmids after 8 h of culture when the medium were exchanged. Twenty-four hours after TNF-α stimulation, i.e. after 72 h culture, the cells and supernatants were harvested, centrifuged, and stored at -80℃ for later use. In the I/R models, four animals in each group were killed at the time points of reperfusion, at 0.5, 2, 6, 12 and 25 h. Blood was taken from the heart, centrifuged at 3200 r/min for 5 min at room temperature, and the serum was separated, frozen and stored at -80℃ for later detection. Simultaneously, some liver tissues were frozen and stored in liquid nitrogen, and other liver tissues were fixed with 4% paraformaldehyde, embedded with paraffin, stained with HE, and observed pathologically under a light microscope.

The TNF-α levels in the supernatants and serum were determined by ELISA, and each sample was duplicated. The serum levels of alanine aminotransferase (ALT) were tested by the velocity method.

Expression of UCP-2 in the cells and liver tissues was detected by using RT-PCR[22]. In brief, total RNA of each sample was extracted, the purity and concentration were assayed by DNA/Protein Analyzer, and the same total RNA concentration in each group was adjusted. cDNA was prepared by reverse transcription. The primer sequences of UCP-2 for PCR amplification were as follows: F, 5'-ATGGTTGGTTCAAGGCCAC-3'; R, 5'-TCATGAGGTTGGCTTTCAGG-3'[23]. The reaction conditions included: 94℃ for 3 min; 94℃ for 30 s,

Table 1 Serum TNF-α levels in I/R mice (pg/mL) at various time points after reperfusion

Group	Serum TNF-α levels				
	0.5 h	2 h	6 h	12 h	24 h
B1	59 ± 7	81 ± 11	153 ± 23	196 ± 35	278 ± 43
B2	106 ± 16[a]	142 ± 24[a]	231 ± 31[a]	316 ± 47[a]	394 ± 52[a]
B3	97 ± 17[a]	151 ± 21[a]	244 ± 27[a]	332 ± 45[a]	401 ± 48[a]

[a]$P < 0.05$ B2 *vs* B1; and B3 *vs* B1.

and 60℃ for 1 min (45 cycles). The products were electrophoresed with 1.5% agarose gel. The sample expression of mRNA was calculated according to the following formula: Relative value of the detected samples $= 2^{(\Delta Ct_{\text{detected sample}}-\Delta Ct\beta\text{-actin})}$; $\Delta Ct = Ct_{\text{negative control}} - Ct_{\text{detected sample}}$.

Statistical analysis

UCP-2 mRNA, TNF-α and ALT levels were expressed as the mean ± SD and analyzed by using SPSS10.0 software. Comparison of the same index in various groups was performed by using ANOVA and the *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

TNF-α levels in cell culture and I/R model

TNF-α levels in A2, A4 and A5 groups differed significantly. TNF-α levels at the five time points after reperfusion in the B1, B2 and B3 groups are shown in Table 1. Results indicated that TNF-α levels in the B2 and B3 groups were significantly higher than that in the B1 group ($P < 0.05$), but there was no significant difference between the B2 and B3 groups.

Expression levels of UCP-2 in cultured hepatocytes and I/R models

Fluorescent quantitative PCR analysis revealed that in the A1, A2, A3, A4 and A5 groups, expression levels of UCP-2 were 0.125 (0.117-0.134), 1.558 (1.548-1.636), 1.079 (1.007-1.157), 2.949 (2.670-3.278) and 0.732 (0.660-0.812), respectively. It was indicated that in the A2 and A4 groups, the expression levels of UCP-2 were significantly higher than in the corresponding non-stimulated groups ($P < 0.05$). The expression level in the A4 group was significantly higher than in any other group, and in the A5 group, UCP-2 expression was significantly reduced as compared with that in the A4 group ($P < 0.05$). In the B1 group, expression levels of UCP-2 in liver tissues at the five time points after reperfusion were 0.252 (0.248-0.255), 0.287 (0.281-0.293), 0.401 (0.389-0.412), 0.432 (0.427-0.438) and 0.470 (0.438-0.503), respectively; those in the B2 group were 0.940 (0.883-1.000), 1.020 (1.016-1.024), 1.309 (1.241-1.382), 1.469 (1.360-1.587) and 1.629 (1.478-1.796), respectively; and those in the B3 group were 0.320 (0.255-0.402), 0.400 (0.333-0.481), 0.530 (0.467-0.602), 0.570 (0.510-0.638) and 0.621 (0.564-0.682), respectively. The expression level in B3 was significantly lower than in B2, which suggests that the expression of UCP-2 was inhibited (Figures 1 and 2).

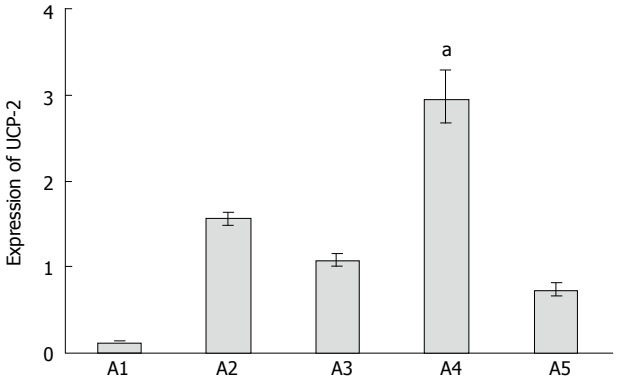


Figure 1 Expression levels of UCP-2 in cell culture. [a]$P < 0.05$ *vs* A5.

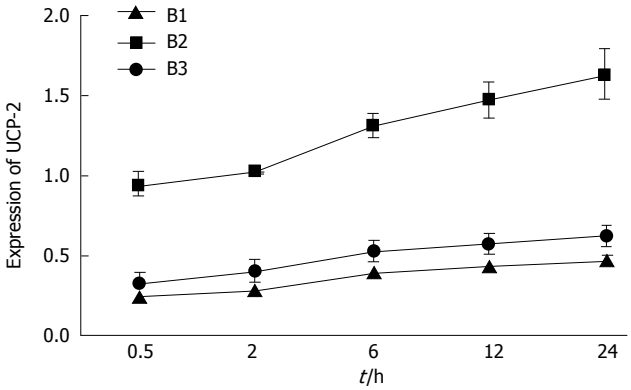


Figure 2 Expression levels of UCP-2 in I/R models.

Serum ALT levels in I/R model mice

In all I/R model mice, serum ALT levels were increased. ALT levels in the B1, B2 and B3 groups were 296 ± 65, 952 ± 147 and 301 ± 74 U/L, respectively. Statistical analysis revealed that ALT levels in the B2 group were significantly higher than those in the other two groups. Among the three groups, B3 had a significantly decreased ALT level after transfection of the plasmids that inhibited expression of UCP-2, which suggests that inhibition of UCP-2 expression can alleviate liver injury.

Pathological examination of liver tissues

In the B1 group, swelling and hydropic degeneration of hepatocytes and hepatic sinusoid endothelial cells, stasis of hepatic sinusoids, and disorder of sinus funiculus were seen. There was exfoliation of partial endothelial cells in the central vein, vacuolar degeneration of hepatocytes on the peripheral zone, and local necrosis in hepatic lobules and at the region adjacent to the portal area. In the B2 group, the above pathological changes were aggravated, and showed multiple local necrosis. However, in the B3 group, the pathological changes in liver tissues were significantly alleviated as compared with those in B2 group (Figure 3).

DISCUSSION

It has been reported that UCP-2 is not expressed in normal liver tissues, but is over-expressed in fatty liver[24].

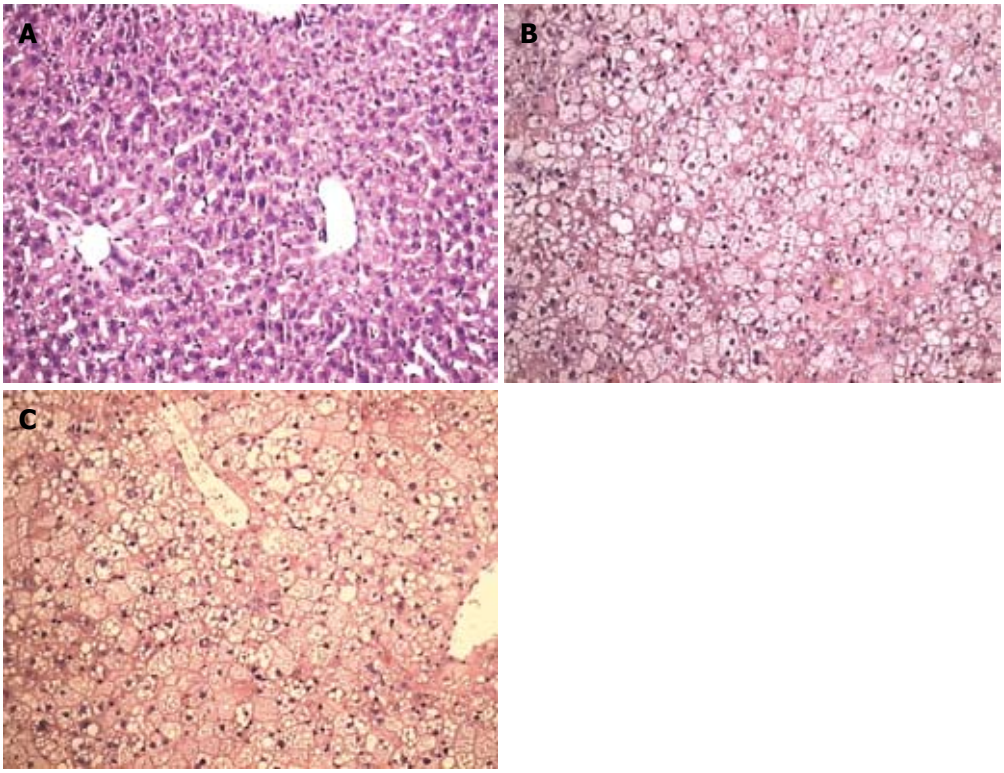


Figure 3 In the B1 group, swelling and hydropic degeneration of hepatocytes and hepatic sinusoid endothelial cells, stasis of hepatic sinusoids, and disorder of sinus funiculus were seen. There was exfoliation of endothelial cells in the central vein, vacuolar degeneration of hepatocytes at the peripheral zone, and local necrosis in hepatic lobules and at the region adjacent to the portal area (HE, × 200); In the B2 group, the pathological changes in Figure 1 were aggravated, showing multiple local necrosis (HE, × 200); In the B3 group, the pathological changes in liver tissues were significantly alleviated as compared with those in B2 (Figure 3B).

Moreover, the abnormal over-expression of UCP-2 in fatty liver can result in a decrease of hepatic ATP content[25,26]. Studies by Chavin *et al* have discovered that in ob/ob mice (approximately 70%-80% of liver lipid content) expression of UCP-2 is four to five times higher than in normal liver tissues, and the ATP content is decreased in mitochondria[27]. In normal conditions, over-expression of UCP-2 does not result in abnormal levels of serum ALT, bile acid metabolites and clotting factors, but under stress conditions, e.g. I/R, over-expressed UCP-2 can lead to severe hepatic cell injury because of ATP reserve deficiency[28,29]. In this study, it was found that in primary culture of fatty liver cells and ob/ob mouse I/R models, expression of UCP-2 was significantly higher than that in normal liver cells and normal mouse I/R models, and TNF-α levels in ob/ob mice were significantly higher than those in normal mice. However, in fatty liver cells stimulated with TNF-α, expression of UCP-2 was significantly higher than that in other cultured cells, which suggests that increased serum TNF-α levels can further enhance the increased expression of UCP-2 in fatty liver tissues. Previous studies have also revealed that TNF-α can induce the expression of UCP-2 in normal hepatocytes and mouse liver tissues, and it is believed that the increased TNF-α is related to lipopolysaccharide (LPS) stimulation[17,30]. Therefore, in I/R, LPS stimulation can result in increased TNF-α levels, which in turn induce higher expression of UCP-2 in fatty liver cells, which causes further decrease of the ATP reserve in hepatocytes, which subsequently aggravates the I/R injury in fatty liver. In this study, the plasmids that inhibited UCP-2 expression were constructed and transfected into hepatocytes and the mouse I/R models, and it was found that expression of UCP-2 mRNA was significantly reduced, which was not related to TNF-α levels. Simultaneously, in the model with transfected mice, serum ALT levels were significantly lower than those in non-transfected mice, and hepatic pathological examination revealed that the I/R injury of liver tissues was alleviated, which suggests that targeting inhibition of UCP-2 expression in fatty liver may protect ob/ob mice from I/R injury. In future studies, the role of UCP-2 expression inhibition in obese mouse liver transplantation will be further investigated.

COMMENTS

Background

Primary graft non-function (PNF) after fatty liver transplantation was related to liver ischemia-reperfusion (I/R) injury during transplantation. Uncoupling protein 2 (UCP-2) has shown a close relationship with I/R injury of fatty liver transplantation by mediating proton leak and interfering with mitochondrial ATP synthesis

Research frontiers

The impact of UCP-2 deficiency during acute liver injury has been investigated recently, and it has been found that lack of UCP-2 reduces Fas-mediated liver injury in ob/ob mice. Although ablation of UCP2 may have no significant impact on steatosis-induced changes in ROS production of Kupffer cells, it alleviates the energy compromise in acutely challenged fatty hepatocytes.

Innovations and breakthroughs

UCP-2 has been reported to be closely related to I/R injury of steatotic liver. However, the direct link between UCP-2 expression and susceptibility of fatty liver to I/R injury is lacking. This study considered UCP-2 as a therapeutic target and investigated its role in fatty liver I/R injury *in vitro* and *in vivo*. The results showed that suppression of UCP-2 expression alleviated I/R injury of fatty liver in mice. This may provide a new gene therapy target.

Applications

The result of this study indicated that UCP-2 could be an effective gene therapy target of fatty liver I/R injury. By suppressing UCP-2 expression, more steatotic liver donors may be used in liver transplantation, and partially alleviate the shortage of donor livers.

Terminology

UCP-2 is an inner mitochondrial membrane carrier. It mediates proton leak and allows the dissipation of the proton electrochemical gradient, which results in the uncoupling of oxidation and ATP synthesis. ROS include superoxide, hydrogen peroxide, and hydroxyl radicals. At low levels, these species may function in cell signaling processes. At higher levels, these species may damage cellular macromolecules.

Peer review

This is a well written and interesting manuscript. The authors demonstrated the alleviation of I/R injury in ob/ob mice by inhibiting UCP-2 expression.

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RAPID COMMUNICATION

Regulation of demethylation and re-expression of RASSF1A gene in gastric cancer cell lines by combined treatment of 5-Aza-CdR and NaB

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Abstract

AIM: To investigate the changes of methylation state and expression of RASSF1A gene in human gastric cancer cell lines SGC7901 and BGC823 which were treated *in vitro* with demethylating agent 5-Aza-CdR in combination with histone deacetylase inhibitor NaB.

METHODS: After SGC7901 and BGC823 cells were treated with 5-Aza-CdR and/or NaB, the methylation state of RASSF1A gene was detected by methylation-specific PCR, and the changes in expression of mRNA and protein level of RASSF1A gene were observed by RT-PCR and Western-blotting before and after drug treatment.

RESULTS: Hypermethylation was detected in the promoter region of RASSF1A gene in both SGC7901 and BGC823 cells, and there was no expression of this gene at both mRNA and protein level. After treatment with 5-Aza-CdR, demethylation occurred in the promoter region of RASSF1A gene, which subsequently induced re-expression of this gene. The treatment with NaB alone showed no effect on the methylation state and expression of RASSF1A gene. The combined treatment of 5-Aza-CdR and NaB induced complete demethylation of RASSF1A gene, leading to a significantly higher re-expression of the mRNA and protein of RASSF1A than those treated with 5-Aza-CdR alone ($P < 0.05$).

CONCLUSION: Hypermethylation in the promoter region is related to inactivation of RASSF1A gene in human gastric cancer cell lines SGC7901 and BGC823, while demethylating agent 5-Aza-CdR can reverse the methylation state of RASSF1A gene and induce its re-expression. Histone deacetylase inhibitor NaB had a synergistic effect with 5-Aza-CdR in both demethylation and gene transcriptional regulation.

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Key words: 5-Aza-CdR; NaB; RASSF1A gene; Gastric cancer; DNA methylation

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INTRODUCTION

Gastric cancer is a malignant tumor which seriously threatens the human health worldwide. It has been shown that the occurrence of gastric cancer is a multi-gene-involved, multi-stage pathological process under the action of both genetic and epigenetic factors, and that epigenetic mechanism plays an important role in the occurrence and development of gastric cancer[1-4]. Various factors can lead to the loss of function of tumor suppressor genes (TSGs). It has been confirmed that DNA methylation is the third, sometime the only mechanism for the inactivation of TSG, besides gene mutation and deletion[5]. The Ras associated domain family gene 1A (RASSF1A) is an established TSG that can inhibit cell growth, and promote apoptosis and senescence through various ways[6-9]. The methylation in the promoter region of RASSF1A gene can cause depletion of RASSF1A expression, thereby leading to occurrence of tumors, which has been confirmed in human gastric cancer[10,11]. Since the regulation of gene expression by abnormal methylation is reversible, demethylating agents could be used to induce re-expression of the inactivated genes due to methylation[12]. Therefore, changing the CpG island state in the gene promoter region is a potential method for clinical treatment of cancers[13]. The demethylating agent 5-Aza-2'-deoxycytidine (5-Aza-CdR) is a cyto-

sine analogue formed by modified C-5 position of pyrimidine, and used as a DNMT (DNA methyltransferase) inhibitor for the clinical treatment of leukemia[14,15]. Histone acetylation may be related to the occurrence of human gastric cancer[16]. DNA methylation and histone acetylation are associated with each other in the epigenetic regulation of gene expression, and they together play an important role in the occurrence of tumors. They affect each other and likely have the synergistic effect[17]. Therefore, the combined treatment of demethylating agent and histone deacetylase inhibitor can induce the gene demethylation, reverse the transcriptional inhibition, and make the gene re-express, which might be used for the clinical treatment of tumors[18].

In this study, the gastric cancer cell lines were treated *in vitro* with demethylating agent 5-Aza-CdR in combination with histone deacetylase inhibitor NaB (sodium butyrate) so as to investigate the changes in methylation state and expression of RASSF1A gene before and after drug treatment, and to further explore the roles of these two drugs in regulating RASSF1A gene expression. The results will provide the experimental evidences for the clinical therapy.

MATERIALS AND METHODS

Materials

The main materials used included human gastric cancer cell lines SGC7901 and BGC823 (Department of Cell Biology of China Medical University), RPMI 1640 medium (Hyclone), 5-Aza-CdR (Sigma), NaB (Sigma), hydroquinone (Sigma), sodium bisulfite (NaHSO3) (Sigma), Wizard DNA Clean-up (Promega), glycogen (New England Biolabs), SssI (New England Biolabs), Taq enzyme (Takara), dNTPs (Takara), DMSO (Sigma), the RT-PCR Kit (TaKaRa), the sheep anti-human RASSF1A polyclonal antibody (Santa Cruz) and the TRIzol reagent for total RNA extraction (GIBCO BRL).

Methods

Cultivation of human gastric cancer cell lines: BGC823 and SGC7901 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum at 37℃ with 50 mL/L CO_2. At the logarithmic phase of cell growth, with a percentage of viable cells over 95%, the cells were remained hungry for 24 h in the RPMI 1640 medium containing 1% fetal bovine serum, followed by the drug treatment. Cells were transferred every day into the fresh medium containing 5-Aza-CdR or NaB at its experimental dosage. In the fifth day of cultivation, cells were harvested for further analysis. The cells without the intervention of medicine were used as the control group.

Experimental groups: (1) 5-Aza-CdR treatment groups at the dosage of 1.0 or 3.0 μmol/L; (2) NaB treatment groups at the dosage of 1.0 or 3.0 mmol/L; (3) Combined treatment groups: 5-Aza-CdR at 1.0 μmol/L in combination with NaB at 1.0 mmol/L, and 5-Aza-CdR at 3.0 μmol/L in combination with NaB at 3.0 mmol/L. The gastric cancer cells were first treated with 5-Aza-CdR at 1.0 or 3.0 μmol/L for 48 h, followed by washing, and then were treated with NaB at 1.0 or 3.0 mmol/L for 72 h; the cells were then harvested for further analysis.

Detection of methylation state of RASSF1A by MSP: Different groups of BGC823 and SGC7901 cells were harvested after five days treated with different drugs, and cellular DNA was isolated from the harvested cells using phenol-chloroform-isoamyl alcohol assay. The concentration and purity of DNA were detected by the UV spectrophotometry. DNA modification and purification were conducted as described previously[19].

Methylation-specific PCR reaction: Total reaction mixture volume of 25 μL, containing 2 μL DNA, 2.5 μL 10 × PCR buffer, 0.5 μL each of the sense and antisense primers, 2 μL dNTPs, 0.2 μL Taq enzyme, and 17.3 μL double-distilled water. Reaction condition: pre-denatured at 95℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 58℃ for 45 s, and 72℃ for 30 s, and with a final extension at 72℃ for 10 min. The annealing temperature was 58℃ for the methylated reaction, while 57℃ for the un-methylated reaction. The methyltransferase SssI-treated and untreated peripheral blood cell DNA from the healthy adults were used as the positive and negative controls respectively, while the double-distilled water as the blank control. The 1000 bp DL2000 was used as the molecular weight markers. The sequences of the methylated primers are as follows: the upstream primer 5'-GTGTTAACG CGTTGCGTATC-3' and the downstream primers 5'-A ACCCCGCGAACTAAAAACGA-3', generating a 94 bp PCR product, while the un-methylated primers are: the upstream primer 5'-TTTGGTTGGAGTGTGTTAATG TG-3' and the downstream primers 5'-CAAACCCCACAA ACTAAAAACAA-3', generating a 108 bp PCR product. The PCR products were subjective to the 2% agarose gel electrophoresis at 100 V for 40 min. The gels were photographed using a laser density scanner (Pharmacia LKB Ultroscan) for analysis. Each experiment was repeated five times.

Analysis of mRNA expression of RASSF1A by semi-quantitative RT-PCR: The cellular total RNA was isolated using the Trizol reagent from the harvested cells. The cDNAs were synthesized from the templates in presence of reverse transcriptase and oligo $(dT)_{20}$ primers. The upstream and downstream RASSF1A mRNA primers were 5'-GGC GTCGTGCGCAAAGGCC-3' and 5'-GGGTGGCTTCT TGCTGGAGGG-3' respectively, generating a 330 bp PCR product at the annealing temperature of 67℃. The β-actin was used as the internal control, and its upstream and downstream primers were 5'-GTGGGGCGCCCCAGGC ACCA-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3' respectively, generating a 498 bp PCR product. Twenty-five μL PCR reaction mixture was pre-denatured at 95℃ for 2 min, and 35 cycles of 94℃ for 40 s, 67℃ for 40 s, and 72℃ for 60S were performed with a final extension at 72℃ for 6 min. The RT-PCR products were subjective to the 2% agarose gel electrophoresis. The gels were photographed using an Alpha Image 2000 automatic formatter, and the amount of each PCR product was analyzed using the Fluorchem V 2.0 Stand Alone software. The scanning

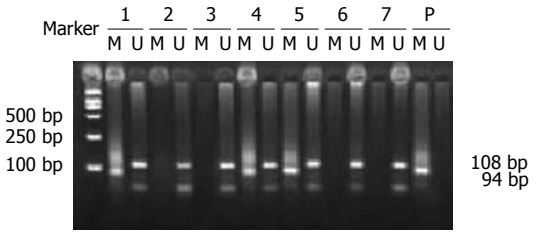


Figure 1 Change in methylation state of RASSF1A gene in gastric cancer cell line SGC7901. Markers: DL2000; 1-7: Control, 5-Aza-CdR at 1.0 μmol/L, 5-Aza-CdR at 3.0 μmol/L, NaB at 1.0 mmol/L, NaB at 3.0 mmol/L, 5-Aza-CdR at 1.0 μmol/L in combination with NaB at 1.0 mmol/L, and 5-Aza-CdR at 3.0 μmol/L in combination with NaB at 3.0 mmol/L; P: Positive control; M: Methylated result; U: Un-methylated result; 1, 4 and 5: Methylation positive, 94 bp; 2, 3, 6 and 7: Methylation negative, 108 bp.

Table 1 mRNA expression of RASSF1A gene in gastric cancer cell lines SGC7901 and BGC823

	1	2	3	4	5	6	7
SGC7901	0	0.29 ± 0.03	0.45 ± 0.05	0	0	0.75 ± 0.04[a]	0.88 ± 0.09[a]
BGC823	0	0.36 ± 0.05	0.64 ± 0.07	0	0	0.82 ± 0.09[a]	0.89 ± 0.05[a]

[a]$P < 0.05$, *vs* groups 2 and 3 ($F_{SGC7901} = 208.83$, $P < 0.001$, $F_{BGC823} = 62.10$, $P < 0.001$).

optical density of each band was taken as the indicator of the gene expression level. Taking the β-actin as the internal control, the ratio of scanning density of RASSF1A gene to that of β-actin was obtained. The ratio values used as the analyzing index were further compared between different experimental groups to investigate the changes in mRNA expression of RASSF1A. Each experiment was repeated five times.

Analysis of protein expression of RASSF1A by Western blotting: 200 μL of cell lysis buffer was mixed with 40 μL harvested cells to isolate the total cellular protein, and then quantitated by the phenol reagent method, 100 μL of the total protein was denaturated for 10 min, and 25 μL was taken for the 10% polyacrylamide gel electrophoresis. The proteins separated by the electrophoresis were then transferred onto the PVDF membrane, and the 10% skim milk used to block the PVDF membrane at room temperature for 1 h. The PVDF membranes were incubated in the 1:200 dilution of the primary antibody at 4℃ overnight, and then in the 1:2000 diluted alkaline phosphatase-labeled secondary antibody solution at 37℃ for 2 h, followed by the DAB coloration. The PVDF membranes were photographed using a ChemiImager 5500 AlPhaInn Ch, and the subsequent data acquisition and mining were conducted with the Fluorchem V2.0 system. Taking the expression level of β-actin protein as the internal control, the ratio of scanning density of RASSF1A protein to that of β-actin was finally calculated and used as the analyzing index to further investigate the changes in protein expression of RASSF1A and compare them between different experimental groups. Each experiment was repeated five times.

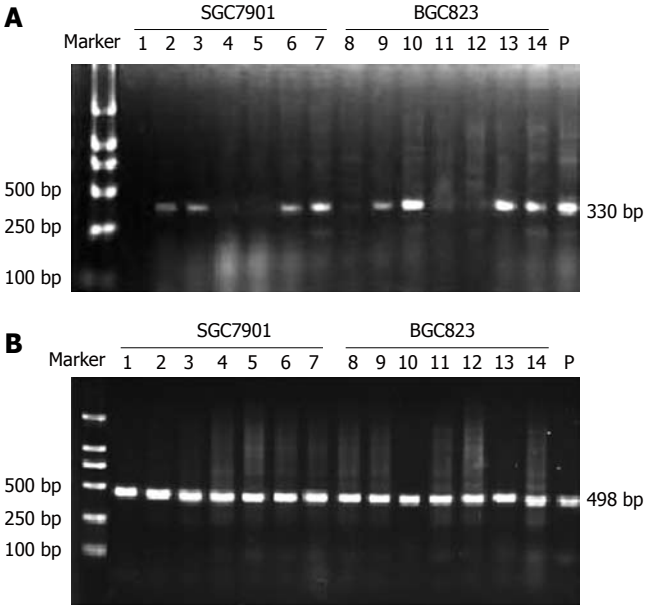


Figure 2 **A**: Change of mRNA expression of RASSF1A gene in gastric cancer cell lines SGC7901 and BGC823. Markers: DL2000; 1-7 and 8-14: Control, 5-Aza-CdR at 1.0 μmol/L, 5-Aza-CdR at 3.0 μmol/L, NaB at 1.0 mmol/L, NaB at 3.0 mmol/L, 5-Aza-CdR at 1.0 μmol/L in combination with NaB at 1.0 mmol/L, and 5-Aza-CdR at 3.0 μmol/L in combination with NaB at 3.0 mmol/L of SGC7901 and BGC823 respectively; P: Positive control, 330 bp; **B**: mRNA expression of β-actin in gastric cancer cell lines SGC7901 and BGC823.

Statistical analysis

Statistical analyses were performed using the SPSS11.5 software package (SPSS, Inc, Chicago, IL). Data were reported as median (minimum-maximum) unless otherwise stated. Continuous variables were compared by the analysis of variance (ANOVA), and multiple comparisons were analyzed by the LSD.

RESULTS

Methylation state of RASSF1A gene

Hypermethylation in the promoter region of RASSF1A gene was detected in gastric cancer cell lines SGC7901 and BGC823, and demethylation appeared after treatment with 5-Aza-CdR for five days, while NaB had no influence on its methylation state. The combined use of 5-Aza-CdR and NaB induced complete demethylation of RASSF1A gene in both SGC7901 and BGC823 cells (Figure 1).

mRNA transcription of RASSF1A gene

There was no mRNA transcription of RASSF1A gene in SGC7901 and BGC823 cells, and it re-expressed after treatment with 5-Aza-CdR for 5 d, while NaB had no influence on its expression. The combined use of 5-Aza-CdR and NaB induced higher re-expression of RASSF1A gene at the mRNA level than the use of 5-Aza-CdR alone ($P < 0.05$) (Figure 2; Table 1).

Protein expression of RASSF1A gene

There was no expression of RASSF1A protein in SGC7901 and BGC823 cells, and it re-expressed after treatment with 5-Aza-CdR for 5 d, while NaB had no

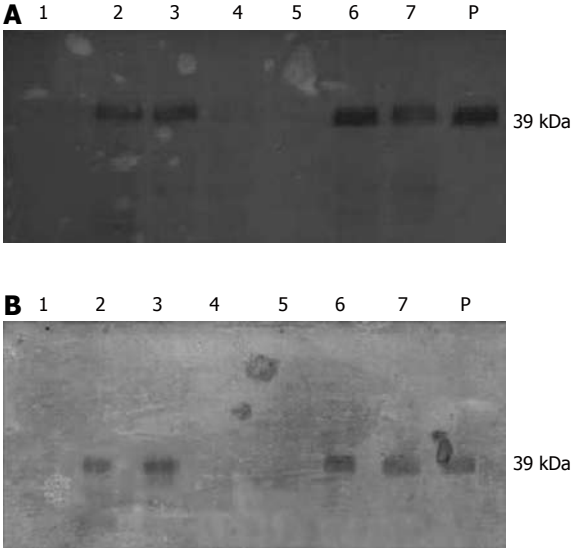


Figure 3 **A**: Change of protein expression of RASSF1A gene in gastric cancer cell line SGC7901; **B:** Change of protein expression of RASSF1A gene in gastric cancer cell line BGC823. 1-7: Control, 5-Aza-CdR at 1.0 μmol/L, 5-Aza-CdR at 3.0 μmol/L, NaB at 1.0 mmol/L, NaB at 3.0 mmol/L, 5-Aza-CdR at 1.0 μmol/L in combination with NaB at 1.0 mmol/L, and 5-Aza-CdR at 3.0 μmol/L in combination with NaB at 3.0 mmol/L; P: Positive control, 39 kDa.

influence on its expression. The combined use of 5-Aza-CdR and NaB induced higher re-expression of the RASSF1A gene at the protein level than the use of 5-Aza-CdR alone ($P < 0.05$) (Figure 3A and B, Table 2).

DISCUSSION

The gene transcriptional regulation in eukaryotes is a complex, multi-step process, and the covalent modification of chromatin participates in the regulation of transcription initiation[20]; it can occur in both DNA and histone. DNA modification is primarily the cytosine methylation, while the covalent modifications of histone include acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation, which thereby constitute the histone code[21]. The occurrence of tumors is a process under the mutual action of genetic and epigenetic mechanisms. The epigenetic molecular mechanisms include DNA methylation, histone modification, chromatin remodeling and RNA interference, all of which play important roles in gene transcriptional regulation[22].

As shown in this study, the action of 5-Aza-CdR on gastric cancer cell lines SGC7901 and BGC823 resulted in the demethylation of RASSF1A gene accompanied by the re-transcription of mRNA and re-expression of protein; this confirmed that 5-Aza-CdR regulated the transcription of RASSF1A gene. DNA methylation regulates gene expression, while the DNA hypomethylation promotes gene expression and DNA hypermethylation inhibits it[5]. 5-Aza-CdR is a cytidine analogue; as a demethylating agent, its mechanism of action is primarily through its covalent bonding to DNMT, which will reduce the biological activity of DNMT, thus lowering the methylation level and regulating gene expression[23]. The demethylation of TSGS by 5-Aza-CdR has been reported[24]. It has been shown recently that the mechanism of action of 5-Aza-CdR is

Table 2 Protein expression of RASSF1A gene in gastric cancer cell lines SGC7901 and BGC823

	1	2	3	4	5	6	7
SGC7901	0	0.66 ± 0.07	0.71 ± 0.09	0	0	0.88 ± 0.06[a]	0.89 ± 0.05[a]
BGC823	0	0.45 ± 0.03	0.65 ± 0.08	0	0	0.78 ± 0.04[a]	0.83 ± 0.06[a]

[a]$P < 0.05$, compared with groups 2 and 3 ($F_{SGC7901} = 14.41$, $P < 0.001$, $F_{BGC823} = 46.01$, $P < 0.001$).

likely to inhibit the activity of DNMT3a and DNMT3b[25]. After the action of 5-Aza-CdR, the transcription inhibitor MBD brake away from the demethylated DNA or the transcription repression complex that originally bonded to MBD, and then form to its activated conformation of chromatic body[26]. Whether the gene expression is re-activated after the demethylation of this gene by 5-Aza-CdR, depended on the following two factors: (1) whether the hypermethylation of the CpG island in the gene promoter region is reversed; (2) whether there is the simultaneous existence of a transcription activator to activate the specific target promoter[27]. For some of genes, 5-Aza-CdR alone is sufficient to induce the activation and expression, which can be used to explain the reversed expression of RASSF1A gene in the gastric cancer cells, as observed in the present study. This is supported by the report by Byun *et al*[10].

With the action of NaB, there was no change in the methylation state and expression of RASSF1A gene in both SGC7901 and BGC823 cells. In the eukaryotes, histone is an important component of the elementary structure of chromatin-nucleosome, of which the "N" terminal can be covalently modified such as acetylation. The acetylation of histone is a reversible dynamic process; the acetylized chromatin was related to transcriptional activation, while the desacetylizad chromatin was related to transcription inhibition[28]. The common mechanism of action of histone deacetylase inhibitors HDACIs is to inhibit the histone deacetylation so as to enhance the acetylation level of histone, by which the gene expression was up-regulated[29]. Among HDACIs, the NaB is a kind of nontoxic short-chain fatty acid that has been proved to inhibit the proliferation of cancer cells *in vivo* and *in vitro*, to induce the apoptosis, to promote the cell differentiation and to block the cell cycle[30,31]. It has the potential for the clinical therapy of malignant cancers such as breast cancer, colorectal cancer, endometrial cancer[32] and so on. It has been shown that[33] HDACIs can raise the acetylation level of histone, and change the chromatic structure, thereby inducing the transcriptional activity of some genes. Selker *et al*[34] reported that another HDACI-TSA (trichostatin A) could selectively down-regulate the methylation level of some genes in Neurosporae, but the use of NaB alone has no effect on the gene expression of RASSF1A, which was supported by Lynch *et al*[35].

In this study, the up-regulation of RASSF1A gene under the combined treatment of 5-Aza-CdR and NaB was significantly higher than that under the use of 5-Aza-CdR alone, indicating that the demethylating agent 5-Aza-CdR and the deacetylase inhibitor NaB had a synergistic effect in transcriptional regulation; this was consistent

with the results by Egger *et al*[36]. As shown previously, both DNA methylation and histone acetylation were closely related to the regulation of gene expression. DNA methylation could induce the acetylation of the local histone, and could decrease the chromatin acetylation level[5]. The methylated CpG-binding protein MeCP2 bonded to chromatin by a methylation-dependent manner; through its transcriptional repression domain, MeCP2 was closely related to the inhibition complex consisting of the pontine proteins Sin3A and HDAC[37]. The methylation of promoter region and the histone deacetylation are synergistic in the inhibition of gene transcription. However, the inhibitive effect of histone deacetylation on gene transcription relies on the number of methylation sites. If the mCpG in the promoter region is at low density, the pathway of histone deacetylation plays a major role in inhibiting gene transcription. Under the condition that the number of methylation sites in the promoter region increased to the threshold value that is large enough to lead to the extensive silence of gene expression, the pathway of histone deacetylation plays a secondary role in inhibiting gene transcription. Moreover, the pathway of DNA methylation will play the leading role in the gene transcriptional regulation, and it can inhibit the gene transcription by the manner independent of the histone deacetylation[38]. The rational explanation for the mechanism of synergistic action of 5-Aza-CDR and NaB is that NaB promotes the formation of the activated conformation of chromatic body with the action of 5-Aza-CDR[39]. Fahrner *et al*[40] demonstrated that DNA methylation could maintain the inhibition state of the key histones in the hypermethylated promoter regions, directly or indirectly through the transcriptional inhibition. And there is inducible demethylation of hMLH1 gene in the RKO colon cancer cell line 12 h after the 5-Aza-CdR treatment, and reverse of the acetylation state of histone 48 h after the treatment. These also demonstrate the leading role of the DNA methylation in the transcriptional regulation.

The RASSF1A gene can inhibit cell growth, and promote apoptosis and senescence[7]. This study showed that the loss of expression of RASSF1A gene in gastric cancer cell lines SGC7901 and BGC823 was caused by the hypermethylation in the promoter region, the use of 5-Aza-CdR could result in the demethylation and the restoration of transcriptional activity of RASSF1A gene, and that NaB and 5-Aza-CdR were synergic in regulating the expression of RASSF1A gene. Hypermethylation of RASSF1A gene is closely related to the occurrence of gastric cancer. As RASSF1A gene promotes apoptosis and is also likely associated with the resistance to platinum chemotherapy, the effect of 5-Aza-CdR in conjunction with NaB on the regulation of RASSF1A expression is of significant value in clinical practice.

COMMENTS

Background

Epigenetic mechanism plays an important role in the occurrence and development of gastric cancer. As a tumor suppressor gene, the Ras associated domain family gene 1A (RASSF1A) has been reported to be hypermethylated in human gastric cancer, causing depletion of RASSF1A expression and occurrence of tumors. Histone acetylation may be related to the occurrence of human gastric cancer too. Since the regulation of gene expression by abnormal methylation is reversible, and DNA methylation and histone acetylation are associated with each other in the epigenetic regulation of gene expression, the combined treatment of demethylating agent 5-Aza-2'-deoxycytidine (5-Aza-CdR) and histone deacetylase inhibitor NaB (sodium butyrate) can induce the gene demethylation and reverse the transcriptional inhibition, and then cause the re-expression of the gene, including RASSF1A gene, which might be used for the clinical treatment of gastric cancer.

Research frontiers

DNA methylation and histone acetylation have been extensively studied in many aspects, especially the use of their inhibitors in laboratory and clinical trial for the treatment of human cancers. In high-risk myelodysplastic syndrome and chronic myelomonocytic leukemia, decitabine, one of the demethylating agents, was testified whether it can be used in clinical therapy.

Innovations and breakthroughs

In this study, the synergistic effect of demethylation and re-expression of RASSF1A gene was tested in human gastric cancer cell lines SGC7901 and BGC823 under the combined treatment of demethylating agent 5-Aza-CdR and histone deacetylase inhibitor NaB, which has laid an experimental foundation for epigenetic therapy in clinical treatment of human gastric cancer.

Applications

As hypermethylation and abnormal histone acetylation of RASSF1A gene may be related to the occurrence of human gastric cancer, 5-Aza-CdR and NaB might has a potential value in clinical treatment of human gastric cancer.

Terminology

Epigenetics is defined as modifications of the genome, heritable during cell division, that does not involve a change in the DNA sequence. Epigenetics includes DNA methylation, histone modification, chromatin remodeling and RNA interference molecularly, which play important roles in gene expression regulation. DNA methylation and histone acetylation are the two most widely studied epigenetic changes which regulate gene expression. DNA hypomethylation and hyperacetylation can up-regulate gene expression, while DNA hypermethylation and hypoacetylation can down-regulate gene expression. DNA methylation and histone acetylation interact closely.

Peer review

Shen *et al* showed that the treatment of two gatric cancer cell lines with a demethylating agent restored the expression of the tumor suppressor gene RASSF1A which was further enhanced by the addition of a histone deacetylase inhibitor. This effect of 5-Aza-CdR in conjunction with NaB on the regulation of RASSF1A expression may have a potential value in clinical practice.

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RAPID COMMUNICATION

Diagnosis and management of autoimmune pancreatitis: Experience from China

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Abstract

AIM: To determine the clinical, radiographic and pathologic characteristics, diagnostic and treatment modalities in patients with autoimmune pancreatitis (AIP).

METHODS: In this retrospective study, the data of patients with diagnosed chronic pancreatitis (CP) between 1995 and 2006 in Chinese PLA General Hospital were included to screen for the cases with AIP, according to the following diagnostic criteria: (1) diagnostic histopathologic features, and abound IgG4-positive plasma cells on pancreatic tissues; (2) characteristic imaging on computed tomography and pancreatography, together with increased serum IgG, γ-globulin levels or presence of autoantibodies; (3) response to steroid therapy. The clinical, radiographic and pathologic characteristics, diagnostic and treatment modalities, and outcome of AIP cases were reviewed.

RESULTS: Twenty-five (22 male, 3 female; mean age 54 years, 36-76 years) out of 510 CP patients were diagnosed as AIP, which accounted for 49% (21/43) of CP population undergoing surgical treatment in the same period. The main clinical manifestations included intermittent or progressive jaundice in 18 cases (72%), abdominal pain in 11 (44%), weight loss in 10 (40%), and 3 cases had no symptoms. The imaging features consisted of pancreatic enlargement, especially in the head of pancreas (18 cases), strictures of main pancreatic duct and intrapancreatic bile duct. Massive lymphocytes and plasma cells infiltration in pancreatic tissues were showed on pathology, as well as parenchymal fibrosis. Twenty-three patients were misdiagnosed as pancreaticobiliary malignancy, and 21 patients underwent exploratory laparotomy, the remaining 4 patients dramatically responded to steroid therapy. No pancreatic cancer occurred during a mean 46-mo follow-up period.

CONCLUSION: AIP patients always are subjected to mistaken diagnosis of pancreatic cancer and an unnecessary surgical exploration, due to its similarity in clinical features with pancreatic cancer. The differential diagnosis with high index of suspicion of AIP would improve the diagnostic accuracy for AIP.

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Key words: Autoimmune pancreatitis; Pancreatic neoplasms; Differential diagnosis; Steroid therapy

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a unique chronic inflammation of the pancreas in which autoimmune mechanisms are involved in the pathogenesis[1-3]. Since Sarles *et al* first described AIP case in 1961[4], increasing numbers of AIP patients have been diagnosed worldwide[5-9]. Although AIP has distinct characteristics at clinical, radiologic, histologic, and serologic findings[1-3,8], due to the extreme similarity in clinical manifestations of pancreaticobiliary malignancies, the benign condition of AIP has frequently been diagnosed or suspected as pancreatic cancer, especially when clinicians have no awareness of AIP, which consequently impose the patients an unnecessary pancreatic resection[10-12].

The diagnosis of AIP is mainly based on the diagnostic criteria proposed by the Japan Pancreas Society (JPS) in 2002[13]. However, these criteria preclude many problematic cases with clinical findings compatible with AIP, as well as with excellent response to steroids, as a result of failure to fulfill the JPS criteria. Recently, some revised or new diagnostic criteria have been proposed[13-15], including the Mayo Clinic Criteria.

In this study, we reviewed 25 AIP patients from a single institution in China, and analyzed the clinical, radiographic and pathologic characteristics, diagnostic and treatment modalities, outcome, especially the encountered lessons, in order to improve the diagnostic accuracy for patients with AIP.

MATERIALS AND METHODS

Diagnostic algorithm and diagnostic criteria for assessment of AIP

The preliminary screening for AIP patients was carried out among 510 patients who were diagnosed as chronic pancreatitis (CP) between January 1995 and June 2006 in Chinese PLA General Hospital. Those who fit the etiology and diagnostic pictures of alcoholic, gallstone, or other recognized causes of classical CP were excluded initially[16], then the diagnosis of AIP was made in patients with $\geqslant$ 1 of these criteria among the remaining patients: (1) diagnostic histopathologic features, and abound IgG4-positive plasma cells on pancreatic tissues; (2) characteristic imaging on computed tomography and pancreatography, together with increased serum IgG, γ-globulin levels or presence of autoantibodies; (3) response to steroid therapy. Above diagnostic criteria derived from the combination of HISORt (histology, imaging, serology, other organ involvement, and its response to steroid therapy) criteria of Mayo Clinic[15] and other diagnostic criteria[11].

Finally, twenty-five patients met the above criteria for AIP, accounting for 4.9% of CP patients in the same time period.

Patients with AIP

The medical records of the 25 patients (22 males, 3 females; mean age 54 years, range 36-76 years) were reviewed. The mean course of onset of AIP is 2-mo (1-14 mo).

Diagnostic modalities for AIP

Ultrasonography and computerized tomography (CT) scanning were performed for all 25 patients, and endoscopic retrograde cholangiopancreatography (ERCP) was performed in 11 patients, and magnetic resonance imaging/magnetic resonance cholangiopancreatography (MRI/MRCP) were done in 7 patients.

Histopathologic examination for AIP

Ultrasound-assisted biopsy specimens of pancreatic lesions were obtained in 6 patients prior to surgery; and intraoperative resected or biopsy specimens of pancreatic lesions were obtained in 21 patients with AIP (2 had both pre- and intra-operative specimens). The specimens were fixed in 10% formaldehyde and embedded in paraffin. The pathologic diagnosis of AIP was made by the hematoxylin and eosin (HE)-stained sections and immunohistochemical staining using CD4, CD8, and IgG4 antibodies.

RESULTS

Clinical manifestations

The main clinical manifestations included intermittent or progressive jaundice in 18 patients (72%), mild epigastric pain or discomfort in 11 (44%), weight loss in 10 (40%), low-grade fever and chronic diarrhea in 2 each. Three patients had no symptoms. Twenty-four percent (6/25) of the AIP patients had a history of autoimmune disease (ulcerative colitis, n = 2; asthma, n = 1; Sjögren's syndrome, n = 1; systemic lupus erythematosus, n = 1; retroperitoneal fibrosis and nephrydrosis, n = 1); other comorbidities included diabetes mellitus (n = 5), chronic acalculous cholecystitis (n = 2), and suspected diagnosis of primary sclerosing cholangitis (n = 2). None of these patients had a history of excess alcohol consumption, or gallstones.

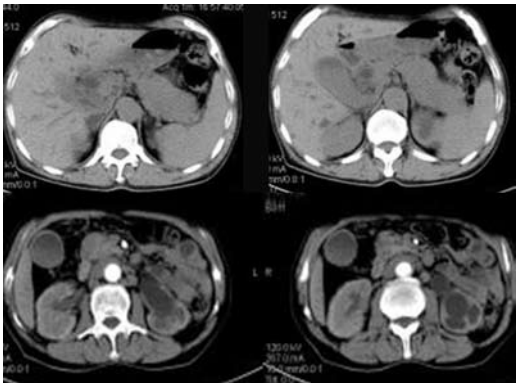


Figure 1 A patient with AIP, CT scan showed a diffusely enlarged pancreas with a sausage-like appearance, together with retroperitoneal fibrosis and nephrydrosis of left kidney.

Laboratory results

Twenty patients (80%) had obstructive jaundice and abnormal serum levels of aminotransferases (ALT and AST) and biliary enzymes (alkaline phosphatase, γ-glutamyltransferase). Hyperglycemia was found in 7 patients (28%), and abnormality of serum CA19-9 level (> 37 U/L) was found in 12 patients (12/19, 63%), among them, the CA19-9 level above 400 U/L in 4 patients. Abnormally elevated levels of serum γ-globulin and IgG were seen in 4 (4/5, 80%). The autoantibodies were determined in 5 patients, only rheumatoid factor, and anti-SSA/-SSB autoantibodies presented in 1 patient each. Mild elevated level of anti-mitochondrial antibody was found in 2. Other antibodies such as antinuclear antibody, antismooth muscle antibody were all negative. No hyperamylasemia was found in all patients.

Imaging features

The results of ultrasonography, CT, and MRI imaging demonstrated that all patients had abnormal alterations in pancreas, massive pancreatic enlargement (sausage-shaped, Figure 1) in 7 patients, and swelling of pancreatic head in 18 patients (Figure 2); the maximal diameter of pancreatic head > 5 cm in 6, and dilation of biliary trees in 18, and thickening of extra-hepatic biliary (Figure 3) or gallbladder walls in 8, retroperitoneal fibrosis and nephrydrosis in 1 (Figure 1).

Ultrasonography revealed a suspected pancreatic cancer in 18 cases (72%), and biliary cancer in 4 cases (16%), and CP was considered in 3. CT scanning showed pancreatic cancer in 15 (60%), and cholangiocarcinoma in 8 (32%), and CP was suspected in 2, for whom, pancreaticobiliary malignancy was not excluded. ERCP and MRI/MRCP

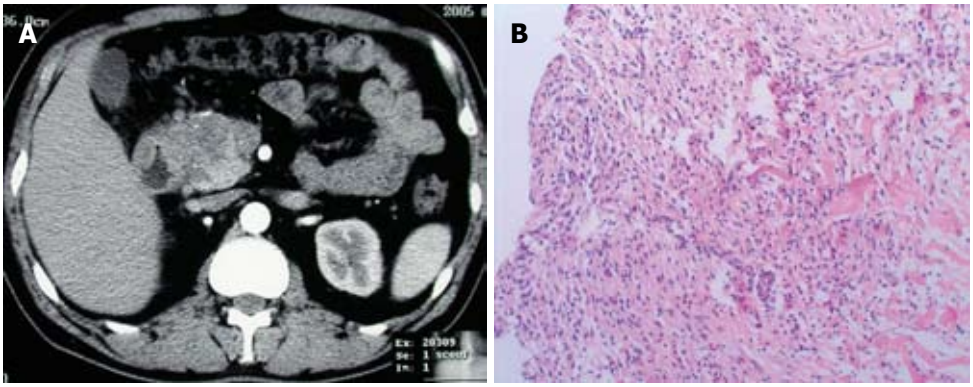


Figure 2 An asymptomatic patient with AIP, CT scan showed an enlarged head of pancreas (**A**), the diagnosis of AIP was confirmed by histologic findings of the pancreatic biopsy specimen: diffuse lymphoplasmacytic infiltration, obliterative phlebitis, dense parenchymal fibrosis and loss of acinar cells (HE, × 100) (**B**). He underwent radiofrequency ablation for the enlarged pancreatic head.

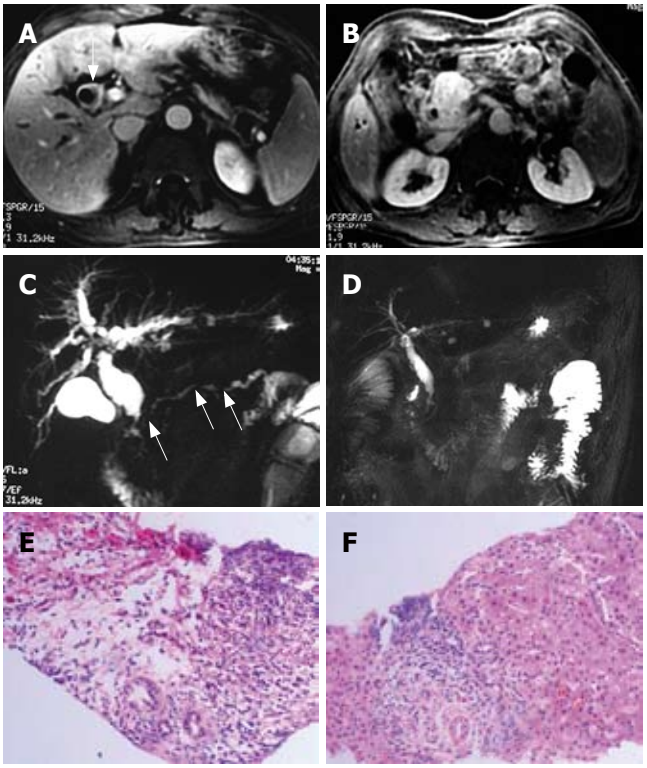


Figure 3 A patient with AIP, MRI imaging before treatment showed dilation of extrahepatic biliary ducts, thickening of hilar biliary wall (arrow) (**A**); and an enlarged mass in the head of pancreas (**B**); MRCP showed dilation of intra- and extra-hepatic biliary ducts, stricture of intrapancreatic and hilar bile duct, and segmental narrowing of main pancreatic duct (arrow) (**C**); after 1 mo of steroid therapy, the radiologic abnormalities resolved (**D**); The histologic findings of the pancreatic biopsy specimen include: dense fibrosis, diffusely lymphoplasmacytic infiltration, and acinar atrophy (HE, × 100) (**E**); the liver biopsy tissue also showed lymphoplasmacytic infiltration, cholestasis, and cloudy swelling of hepatocytes, especially at the portal area (HE, × 200) (**F**).

were detected in 11 cases and 7 cases, respectively, all revealed a stricture of intra-pancreatic common bile duct (CBD) with dilation of upstream biliary trees, focal stricture of hilar duct in 5; multiple stricture of main pancreatic duct (MPD) in 8, and segmental strictures of MPD with minimal upstream dilation in 6 (Figure 3), near-normal configuration of MPD in 2, and poor visualization of MPD in 2.

Based on the clinical and imaging features, a diagnosis or suspected diagnosis of pancreatic cancer and biliary cancer were made in 15 and 8 cases, respectively, and with a 92 percent of misdiagnosing rate at admission.

Histopathologic findings

During surgery, the pancreas was "rock-hard" or firm at palpation, as well as peripancreatic inflammatory adhesion, for whom pancreatic cancer was not ruled out. No calcification, mucoprotein plugs, or pseudocysts was exhibited.

Histological examinations show a significant presence of lymphocytes, plasma cell infiltration surrounding medium and large interlobular ducts, obliterative phlebitis, and scattered eosinophils, as well as acinar atrophy and dense fibrosis in all specimens (Figures 2 and 3). In specimens of 8 cases, dense fibrosis completely replaced the acinar cells and islets, with loss of figuration of pancreatic tissue. Immunohistochemical staining revealed a predominance of CD8+ and CD4+ T lymphocytes, with few B lymphocytes, as well as abundant IgG4-positive plasma cells.

Above-mentioned pathologic features are also present in biliary duct, gallbladder, and liver (Figure 3). In the liver specimens, cholestasis, diffusely cloudy swelling of hepatocytes, and prominent lymphoplasmacytic infiltration are detected, especially at the portal area.

Surgical procedures

Twenty-one patients with AIP underwent 22 sessions of exploratory laparotomy, accounting for 49% (21/43) of the patients with diagnosed CP who undergoing laparotomy: 4 cases underwent pancreaticoduodenectomy (PD) owing to suspicion of pancreatic cancer; 14 cases underwent choledochojejunostomy, among them, 1 case performed 2 sessions of laparotomy (the first laparotomy at a local hospital revealed a firm palpation of pancreatic head and portal vein encasement, and a presumptive diagnosis of advanced pancreatic cancer was made, together with a biliary external drainage; 4 mo later choledochojejunostomy was performed in our hospital after pathologic diagnosis of AIP); for the 3 asymptomic patients, 1 with preoperative diagnosis of AIP insisted on surgery and thus radiofrequency ablation for the enlarged pancreatic head was performed, another 2 only performed intraoperative biopsy of pancreatic tissues. Nineteen of 20 patients with obstructive jaundice underwent surgery, because of the mistaken diagnosis of pancreaticobiliary cancer. Four patients with pathologic diagnosis of AIP through ultrasound-guided percutaneous pancreatic biopsy did not undergo laparotomy.

Treatment and follow-up results

Four patients take oral prednisolone, and repeated

CT and/or MRI revealed improvement of pancreatic imaging 1 mo after steroid therapy (Figure 3), the clinical manifestation and serum levels of γ-globulin, IgG, and CA19-9 restored normally. Furthermore, the effective response to steroid therapy confirmed the diagnosis of AIP for the suspected cases. After steroid therapy, the abnormality of biliary duct and pancreatic duct normalized, and the nephrydrosis improved significantly, but not the retroperitoneal fibrosis.

All the patients with AIP were followed-up, with a mean period of 46 mo (12-142 mo), and no pancreaticobiliary cancers were progressed, nor the symptoms of jaundice, abdominal pain, or acute episode of acute pancreatitis.

DISCUSSION

Autoimmune pancreatitis (AIP) is a distinct subtype of CP. AIP occurs in the absence of gallstones, pancreas divisum, alcohol abuse, or other factors commonly associated with CP. AIP is characterized histologically by a diffuse lymphoplasmacytic infiltration and dense fibrosis. AIP was first described in 1961 by Sarles *et al*[4], who reported 2 patients with hypergammaglobulinemia. The concept of AIP was first proposed by Yoshida *et al* in 1995[1]. Hereafter, the concept of AIP has been widely accepted, and its clinical, histologic, radiographic, and serologic features have been characterized[5-12]. The cellular mechanisms and genetic factors contributing to AIP are also investigated[17-19].

The following characteristics of AIP have been noted[5-12]: (1) predominance of elder male, with mean age of 55-63 years; (2) nonspecific abdominal pain, painless jaundice (> 60%), and, rarely, acute attack of pancreatitis; (3) dense fibrosis with prominent lymphoplasmacytic infiltration in pancreatic lesions; (4) diffuse or focal irregular narrowing of the MPD on ERCP, together with stricture of intrapancreatic common bile duct and dilation of the bile duct upstream; (5) diffuse or focal enlargement of the pancreas; (6) increased serum γ-globulin or IgG or IG4 levels; (7) the presence of autoantibodies; (8) frequent presence of cholestatic liver dysfunction, hyperbilirubinemia, and elevated CA19-9 level; (9) usually absence of pancreatic calcification and cysts; (10) extra-pancreatic organs involvement in autoimmune diseases, such as ulcerative colitis, Sjögren's syndrome, sclerosing cholangitis; and (11) effectiveness of steroid therapy. Sometimes, AIP patients presented with nephrydrosis or upper gastrointestinal bleeding[20].

Nevertheless, the clinical manifestations of AIP are nonspecific, which makes it difficult to differentiate AIP from pancreaticobiliary cancer. Therefore, a significant number of patients with AIP receive operative treatment for an inflammatory mass suspicious of pancreatic cancer. The incidence of AIP ranges 1.86%-6.6% of CP[21], however, the AIP patients undergoing PD due to a clinical suspicion of a malignancy ranges 21%-28% of CP patients receiving surgery[10,22,23], the AIP lesions sometimes have been identified as unresectable tumor because of vessel infiltration.

In China, few cases with AIP have been reported[9]. In our experience, the AIP patients accounted for 5% of CP population; however, 84% (23/25) of the AIP patients was diagnosed or suspected of pancreaticobiliary malignancy, which resulted in 22 episodes of laparotomy for 21 AIP cases, accounting for 49% (21/43) of CP patients receiving surgical treatment.

The pathological features of lymphoplasmacytic infiltration and fibrosis, acinar atrophy are often used as the gold standard for the diagnosis of AIP[11,24-26]. However, the diagnosis made on pancreatic biopsy specimens may be inconclusive (false negative) or suggestive, because of small sample size and sampling error[26,27]. For those with suspected pancreatic cancer, the role of needle or core biopsy specimen is primarily to exclude malignancy[24]. Although studies have revealed that IgG4-positive plasma cells may be a useful marker for the diagnosis of AIP[28-32], whether IgG4-positive staining can diagnose AIP with certainty needs further investigation[30-33].

Abdominal CT findings include a focally or diffusely pancreatic involvement, especially in the head of pancreas, with low-attenuation or an isoattenuation. Thus, the differential diagnosis of focal AIP from pancreatic cancer is difficult on CT findings only. On CT and MRI, the diffusely enlarged pancreas shows a sausage-like appearance, as well as a peripheral rim of the hypointense "halo". The hallmark findings on ERCP are focal, segmental or diffuse irregular narrowing of the MPD, the upstream duct is minimally dilated. Occasionally, a double duct sign (concomitant presence of distal CBD and distal pancreatic duct stricture, suggesting a tumor in the head of pancreas) may be present. Endoscopic ultrasound in the diagnosis of AIP has not been well characterized; however, endoscopic ultrasound guided fine needle aspiration biopsy is beneficial to establish a diagnosis of AIP and exclude cancer[34].

MRCP poorly shows pancreatic duct strictures[35], but it can well demonstrate stricture of the intrapancreatic bile duct, and dilatation of the upper biliary tract. Sclerosing changes of the extrapancreatic bile ducts similar to PSC are sometimes observed. Steroid therapy is effective for changes of the biliary and pancreatic ducts. In view of its above-mentioned advantages and its noninvasiveness, MRCP can partially replace the role of ERCP in diagnosing of AIP[9].

AIP patients frequently exhibit positive serologic antibodies, and elevated levels of γ-globulin or IgG. It has reported that elevated levels of the IgG4 were a sensitive and specific marker of AIP[36,37]; however, elevated level of IgG4 seems nonspecific for AIP[13,38] and its role requires further study. In China, the determination of serum IgG4 level is still unavailable for most of hospitals, so we did not use it in our diagnostic criteria.

Current diagnostic criteria include the JPS criteria[13,39], the Kim criteria[14], the HISORt criteria of Mayo Clinic[15], and the Italian criteria[13]. This absence of integrated criteria makes it difficult to compare studies from different centers. The problem of JPS criteria is that the cases showing an excellent response to steroids may yet fail to fulfill the JPS criteria[13,39,40]. Thus other 3 criteria include the criterion "response to steroid therapy"[8,13-15]. The dramatic effectiveness of steroid for the suspected AIP cases strongly supports the diagnosis, thus avoiding unnecessary surgery.

Steroid therapy is effective for AIP. The initial dose of prednisone should be 30-40 mg daily for a week, followed

by a taper of the daily dose by 2.5-5 mg per week. The response to steroids is often dramatic[13-15,39]. During the first 2-4 wk of therapy, CT or MRI/MRCP should be used to monitor the response. Laboratory, and functional abnormalities may also show improvement with steroid therapy[13-15,39,41]. A poor response to 1-2 wk of steroid therapy should raise the question of pancreatic cancer or other forms of CP. Surgery is preserved for those in whom the pancreaticobiliary malignancy could not be excluded or who complicated with infection of biliary tract.

In summary, the establishment of diagnosis of AIP should rely on combinations of the clinical, radiographic, histologic and serologic evidence, and a response to steroids. AIP is frequently misdiagnosed as pancreatic cancer, because of the clinical similarity with cancer. In order to improve the diagnostic accuracy for AIP, the clinicians must have a high index of suspicion of AIP and be conversant with its manifestations.

COMMENTS

Background

Autoimmune pancreatitis (AIP) is a unique chronic inflammation of the pancreas. AIP has distinct characteristics at clinical, radiologic, histologic, and serologic findings. However, due to the extreme similarity in clinical manifestations of pancreaticobiliary tumors, the patients with AIP frequently were misdiagnosed as pancreatic cancer, and were imposed an unnecessary pancreatic resection.

Research frontiers

The establishment of reasonable diagnostic criteria compatible with all AIP patients is the major area of research, because the criteria can spare the AIP patients unnecessary pancreatic resection.

Innovations and breakthroughs

Although presence of established diagnostic criteria, that clinicians have the knowledge of AIP and with a high index of suspicion is the prerequisite for accurate diagnosis of AIP. In this retrospective study, we demonstrated that there is a terrible frequency of misdiagnosis and unnecessary surgery in AIP patients if the clinicians have no awareness of AIP.

Applications

The diagnostic accuracy for AIP will be improved, if the clinicians have a high index of suspicion of AIP and are conversant with its manifestations. The accurate diagnosis is mainly based on clinical, radiologic and histologic findings, sometimes together with excellent response to steroid therapy.

Terminology

AIP is a type of chronic pancreatitis characterized by an autoimmune inflammatory process in which prominent lymphoplasmacytic infiltration and fibrosis of the pancreas.

Peer review

This is the first English literature reporting AIP patients from China, most of the AIP patients in China were misdiagnosed and experienced subjected to unnecessary surgery, as their Western counterparts before the entity of AIP was not recognized widely. The diagnostic criteria used in this study are practical for AIP patients from developing countries, where the determination of serum IgG4 is unavailable.

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RAPID COMMUNICATION

Expression of SOCS-1 in the liver tissues of chronic hepatitis B and its clinical significance

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Abstract

AIM: To study the expression of suppressor of cytokine signaling-1 (SOCS-1) in the liver tissues of chronic hepatitis B (CHB) and the clinical significance of this expression.

METHODS: The expression of SOCS-1 in liver tissues of 45 cases of CHB was investigated by immunohistochemical staining, and its correlations with inflammation grades and fibrosis stage were analyzed by SPSS statistics software.

RESULTS: The result showed SOCS-1 expressing could be observed in the liver tissue of CHB. The expression of SOCS-1 was mainly distributed near the portal area in the liver tissue of mild inflammation CHB group, and was diffusely distributed in the liver tissue of moderate and severe inflammation groups. SOCS-1 positive stains mainly appear in the hepatocytes, only a few of liver interstitial cells were involved. Inside the hepatocyte, SOCS-1 positive stains are mainly distributed in the plasma. Some of the staining was observed on the membrane. The inclusion bodies in the plasma of hepatocytes were observed occasionally. There were both obvious correlations between the expression of SOCS-1 and the inflammatory grade, and that between the expression of SOCS-1 and the fibrosis stage.

CONCLUSION: The distribution of SOCS-1 in the liver tissue of CHB is variable. This expression was correlated with the inflammation grade and fibrosis stage.

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Key words: Suppressor of cytokine signaling-1; Janus kinase-signal transducer and activator of transcription; Chronic hepatitis B; Inflammation; Fibrosis

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INTRODUCTION

Hepatitis B virus (HBV) infection is a leading cause of chronic liver diseases globally. The exact mechanism by which HBV induces liver damage is still unknown. The suppressor of cytokine signaling (SOCS) family, which consists of SOCS-1 to 7 and cytokine-inducible SH2-containing protein (CIS), has been proved in the negative feedback regulation of janus kinase-signal transducer and activator of transcription (JAK/STAT) signal transduction pathway, which is induced by cytokine signaling[1-4]. SOCS-1 has been implied in the inhibition of inflammation of human body[5-8]. It also influences the inflammation and fibrosis of liver[9-11]. The recent research data also showed that SOCS-1 negatively regulated the response of chronic hepatitis C (CHC) to a-interferon (IFN-α)[12,13]. Methylation of SOCS-1 is implicated to be correlated with carcinoma, including hepatocarcinoma, pancreatic cancer, multiple myeloma and myeloid leukemia[14-19]. Expression of SOCS-1 was regulated by many factors. Cytokines induced by inflammation upgrade the expression of SOCS-1[20-23]. Recent research data showed that, SOCS-1 transcripts in the livers of CHC were significantly higher than controls[12]. Therefore the objective of this study was to explore weather and how SOCS-1 expression in the liver tissues of chronic hepatitis B (CHB) patients and its relationship with inflammation grade and fibrosis stage of the livers.

MATERIALS AND METHODS

Production of the specimen

Biopsied liver specimens were obtained from 45 CHB patients (44 males and 1 females) hospitalized in the infectious department of the third affiliated hospital of Sun Yat-San University to accept liver biopsy from August 2005 to November 2006. All cases were stratified into 3 groups (G2 to G4, 15 cases each group) according to the modified criteria for grading and staging of chronic hepatitis from hepatitis meeting consensus in 2000 of china.

All cases were HBsAg positive. Patients with infection of hepatitis virus of A, C, D, E, malignancy or antivirus therapeutic history were excluded.

Reagent and antibodies

Rabbit anti-human SOCS-1 monoclonal antibody (sc7006) was purchased from Santa Cruz Biotechnology. Diaminobenzidine (DAB) and Streptavidin Biotin Complex (SABC) were purchased from BOSTER Biotechnology in Wuhan.

Collections of clinical data

The clinical data including age, gender, clinical history, liver function tests results done from 3 d before to 3 d after the biopsy and the evaluation results of liver pathology accorded to the modified criteria for grading and staging of chronic hepatitis were collected for analysis.

Immunohistochemistry

The routine paraffin-embedded liver tissue specimens were used. Antigen retrieval was performed by microwaving for 10 min at 90℃ using Dako target retrieval solution (Dako, Carpinteria, CA, USA)[24]. The immunohistochemistry associated with anti-SOCS-1 was performed as described previously[25]. For a negative control, the primary antibody was replaced with non-immune normal mouse IgG.

Evaluation of the positive results of immunohistochemistry staining

The immunohistochemistry staining was evaluated by the staining area and density as described previously[26]. For the staining area, specimens without staining area score 0. Specimens with staining area smaller than 1/3 of the area observed score 1. The one larger than 2/3 scores 3. That from 1/3 to 2/3 score 2. For the staining density, light staining ones score 1, dark staining ones scored 2. The final score was the sum of area score and density score.

Statistical analysis

The SPSS 13.0 was used for the statistical analysis. Rank test was used to analyze the category rank data. Spearman non-parameter correlation analysis was used to analyze the rank associated data. Probabilities of $P < 0.05$ were assumed as statistically significant.

RESULTS

The distributing mode of SOCS-1 in hepatic acinus

Under microscope, the positive staining for SOCS-1 was observed focally distributed. In the liver tissue of mild CHB group, the positive staining is mainly distributed near the portal area (Figure 1A). In the moderate and severe group, the positive staining is diffusely distributed (Figure 1B).

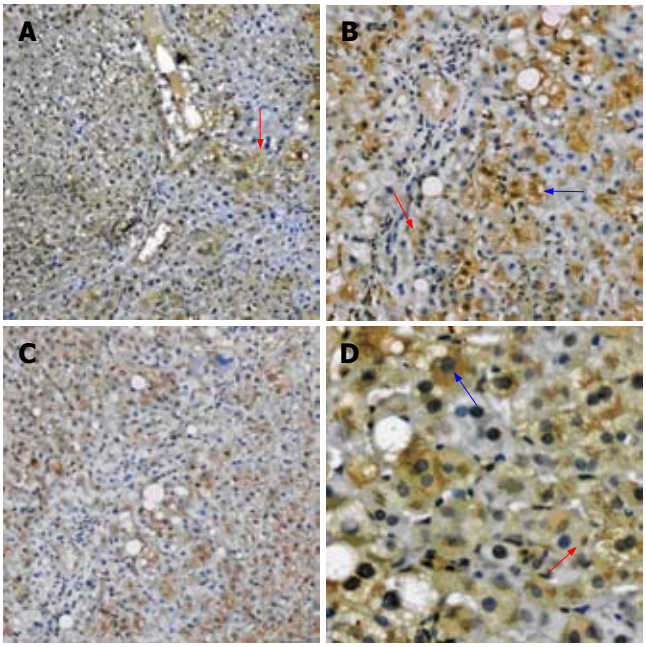


Figure 1 **A**: The liver tissue of mild CHB show the positive staining mainly distributed near the portal area (red arrow); **B**: The liver tissue of severe CHB show the positive staining is diffusely distributed; **C**: The positive staining mainly involves the hepatocytes (blue arrow), only a few of liver interstitial cells are involved (red arrow); **D**: The positive staining is mainly distributed in the plasma (blue arrow), some on the membrane. occasionally in the inclusion bodies (red arrow). (**A**, **B**: Immunohistochemistry SABC × 100; **C**: Immunohistochemistry SABC × 200; **D**: Immunohistochemistry SABC × 400).

Table 1 Evaluation of immunohistochemistry with SOCS-1 in the livers of CHB

		Evaluation of immunohistochemistry					
		0	2	3	4	5	Up
Group	G2	2	2	5	6	0	15
	G3	0	1	8	3	3	15
	G4	0	0	2	7	6	15
	Up	2	3	15	16	9	45

The expression mode of SOCS-1 in liver cell

The positive staining for SOCS-1 mainly involves the hepatocytes, only a few of liver interstitial cells are involved (Figure 1C). The positive staining for SOCS-1 observed in the involved cell is mainly distributed in the plasma. Some of the staining was observed on the membrane. The inclusion bodies in the plasma of hepatocytes were observed occasionally (Figure 1D).

The expression extent of SOCS-1 in hepatic tissue of CHB

Evaluation of the staining for SOCS-1 in the mild hepatitis B group shows that 2 cases scored 0, 2 cases scored 2, 5 cases scored 3 and 6 cases scored 4. Evaluation of the staining for SOCS-1 in the moderate hepatitis B group shows that 1 case scored 2, 8 cases scored 3, 3 cases scored

Table 2 Correlation between the expression of SOCS-1 and liver pathology

		Grade	Stage
SOCS-1 scores	Correlation	0.493	0.308
	P value	0.001	0.04
	n	45	45

4, 3 cases scored 5. Evaluation of the staining for SOCS-1 in the severe hepatitis B group shows that 2 cases scored 3, 7 cases scored 4, 6 cases scored 5 (Table 1).

The correlation between the expression of SOCS-1 and liver pathology

The Spearman correlation analysis was used to analyze the relationship between the expression of SOCS-1 and the pathologic degree. The result showed that the expression of SOCS-1 is positive correlated with the inflammatory degree and extent of the fibrosis stage ($P = 0.001$ and $P = 0.04$) (Table 2).

The correlation between the expression of SOCS-1 and the liver function tests results

The correlation between the expression of SOCS-1 and the liver function tests was analyzed by Spearman correlation analysis. The results showed that no correlation was observed (Table 3).

Table 3 Correlation between the expression of SOCS-1 and the liver function tests results

	mean ± SD	With SOCS-1	
		Correlation	*P*-value
ALT (U/L)	102.78 ± 108.302	-0.077	0.617
AST (U/L)	86.89 ± 77.479	0.081	0.598
ALP (U/L)	82.11 ± 26.551	-0.293	0.051
GGT (U/L)	137.47 ± 256.025	0.133	0.386
ALB (G/L)	42.276 ± 4.7947	-0.058	0.704
GLB (G/L)	29.520 ± 4.1241	-0.13	0.393
TBIL (mol/L)	22.724 ± 20.4962	-0.084	0.583
DBIL (μmol/L)	7.950 ± 8.3926	-0.008	0.961
CHE (U/L)	5771.36 ± 2350.354	-0.026	0.86

DISCUSSION

The exact mechanism of CHB has not been clarified completely. By far, we have known that both the replication of HBV and the immune response of the host play important roles in hepatitis B. SOCS-1 is an important suppressor of cytokine signaling pathway in the serial reactions of immune response of host. SOCS-1 transcripts have been found in thymus cells, lung, liver, spleen, testis, and peripheral blood monocytes of mouse and human being by polymerase chain reaction (PCR)[12,21,27].

Imanaka K compared the expression of SOCS-1 in the liver tissues of 21 cases of CHC patients and 9 control patients by quantitative reverse transcription-polymerase chain reaction (RT-PCR), and found that the transcripts of SOCS-1 in the liver tissues of CHC were significantly higher than controls[12]. The expression of SOCS-1 in the livers of CHC patients implied that it might associate with the inflammation and fibrosis of the livers, yet little has been known about whether and how it expresses in the liver tissues of CHB patients and its relationship with the mechanism of CHB.

The findings herein showed that there were variable expressions of SOCS-1 in the liver tissue of CHB. The expression of SOCS-1 was focally distributed. It was mainly distributed near the portal area in the livers of mild inflammatory CHB patients, and was diffusely distributed in the moderate and severe ones. As we know, inflammatory injure of the livers of CHB patients was mainly distributed near the portal area in the livers of mild inflammatory CHB patients, and was diffusely distributed in the moderate and severe inflammatory CHB patients. The findings suggested that expression of SOCS-1 in the livers of CHB patients accorded with the inflammation of the livers. This indicated that the expression of SOCS-1 was more or less correlated with the inflammation.

Research showed that SOCS-1 is expressed mainly in the hepatocytes and rarely in the interstitial cell. It may be due to the fact that inflammation in the livers of CHB patients mainly involves the hepatocytes. The signaling pathway in the hepatocytes was especially active, so that the expression of SOCS-1 in these cells was elevated. This finding showed that distribution of SOCS-1 in the livers of CHB patients was mainly in hepatocytes due to the influences of inflammation.

The molecular biological data shows that SOCS-1 is synthesized and executes its biological function in the cell plasma. Our results showed that no SOCS-1 was found in the nucleolus and the positive staining mainly seen in hepatocytes plasma. This indicated our findings complied with the previous reports.

Large amount of experiments show that SOCS-1 was an important inhibitor of inflammation. It inhibits the signaling pathway of many cytokines such as interleukin-2 (IL-2), IL-3, IL-4, IL-6, IFN-α, IFN-β, IFN-γ, tumor necrosis factor-α (TNF-α) and so on[21-23].

Naka *et al* found that mice lacking SOCS-1 would die within 3 wk of birth with extensive fatty degeneration of the liver and monocytic infiltration of several organs. His experiment demonstrated that SOCS-1 inhibited the inflammation[28].The new born mice with injection of large amount of IFN-γ showed the same phenotype with mice lacking SOCS-1, which could be contradicted by injection of anti-IFN-γ antibody. It suggested that the hypersensitivity of the mice to IFN-γ caused that inflammatory syndrome. SOCS-1 was a key negative regulator of gamma interferon action *in vivo*[29] and can inhibited the extent of inflammation.

The recent finding also showed that SOCS-1 protected oligodendrocytes from demyelination effects of interferon-gamma[30].

On the other hand, expression of SOCS-1 has been demonstrated to be induced by IFN-γ, Erythropoietin (EPO), Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte-macrophage colony stimulating factor (GM-CSF), IL-2, IL-3, IL-4, IL-6, Leukemia inhibitor factor

(LIF), growth hormone (GH), prolactin (PRL) and so on[20-23]. It referred to that inflammation could induce expression of SOCS-1.

The finding herein showed that expression of SOCS-1 was correlated with the inflammation grade of CHB patients. The reason might be that the inflammatory injure in the livers of the CHB patient induced the expression of SOCS-1. While SOCS-1 was not large enough to inhibit the inflammatory injure that have been occurred. But no significant relation between the expression of SOCS-1 and the liver function tests was observed. It may due to that liver function didn't always accord with the pathological changes.

There were some reports that SOCS-1 can also inhibit liver fibrosis of the CHB patients. Yoshida examined SOCS-1 gene methylation in more than 200 cases of patients with chronic liver diseases and found that the severity of liver fibrosis is strongly correlated with SOCS-1 gene methylation[10]. Our finding showed that expression of SOCS-1 was correlated with the fibrosis stage of CHB patients. But the correlation is low and it is not easy to tell if it affects the progression of fibrosis directly or indirectly through negatively inhibiting the extent of inflammation by our results. Further research is needed to concern the relation between SOCS-1 expression and liver fibrosis.

COMMENTS

Background

Hepatitis B virus (HBV) infection is a leading cause of chronic liver diseases globally. The exact mechanism by which HBV induces liver damages is still unknown. The suppressor of cytokine signaling (SOCS) family, which consists of SOCS-1 to 7 and cytokine-inducible SH2-containing protein (CIS), has been proved in the negative feedback regulation of JAK/STAT signal transduction pathway, which is induced by cytokine. SOCS-1 was discovered by three different groups in 1997. It was named according to its feedback regulation of the cytokine signaling pathway. The former studies showed that SOCS-1 inhibited inflammation, fibrosis and carcinoma, and negatively regulated the response of chronic hepatitis C to IFN-α. It can be upgraded by cytokines induced by inflammation. But little has been known about whether and how SOCS-1 expresses in the liver tissues of CHB patients and its relationship with the mechanism of chronic hepatitis B (CHB).

Research frontiers

There has been a considerable interest over recent years in the significance of SOCS-1 in signaling pathway. The interactions between SOCS-1 and some cytokines have been well explored. And the effect of SOCS-1 on inflammation and malignancy were the hotspots.

Innovations and breakthroughs

We described the distribution of SOCS-1 in the liver tissue of CHB by immunohistochemistry and performed correlation analysis on the expression of SOCS-1 with the inflammatory grade as well as the fibrosis stage and found positive correlation in both for the first time.

Applications

The research stated the distribution of SOCS-1 in the liver tissue of CHB. It is significant for the further statement of the mechanism of CHB and helpful for improvement of the management of CHB.

Terminology

SOCS-1: SOCS-1 was discovered by three different groups in 1997. It was named according to its feedback regulation of the cytokine signaling pathway; 2. JAK/STAT signal transduction pathway: JAK/STAT signal transduction pathway is a signaling conduction pathway. It consisted of JAK and STAT. The mechanism includes phosphorylation of JAK, activation and aggregation of STAT.

Peer review

The paper seems innovative. Zhao *et al* investigated the expression of SOCS-1 in the liver of patients chronically infected with HBV by immunohistochemistry and performed correlation analysis on the expression of SOCS-1 with the inflammatory grade as well as the fibrosis stage and found positive correlation for the first time. The experiment was well designed and plentitude of clinical data. Some interesting observations were obtained. All of these are significant for the further statement of the mechanism of CHB.

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RAPID COMMUNICATION

Quality of care for patients with inflammatory bowel disease in East China

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Abstract

AIM: To investigate the quality of care for a hospital based-cohort of patients with inflammatory bowel disease (IBD) from East China according to the current practice guidelines.

METHODS: A retrospective review was conducted, involving 177 patients with IBD admitted to Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University between June 2000 and June 2006. Data regarding demographic and clinical characteristics as well as medical therapy including use of oral aminosalicylates, topical therapy, corticosteroid agents, immunomodulatory agents (such as azathioprine) at admission and outpatient clinic visit were analyzed.

RESULTS: A total of 177 eligible patients were evaluated in this study, including 71 patients with Crohn's disease (CD) and 106 with ulcerative colitis (UC). All were the Han nationality Chinese with active disease at baseline. All the 106 patients with ulcerative colitis received optimal doses of aminosalicylate while 27 of 68 (39.7%) patients with ileal or colonic CD received the suboptimal doses of aminosalicylate. The incidence of suboptimal dose of aminosalicylate was significantly higher in CD patients with small intestine involvement only (52.8% *vs* 25.0%, $P = 0.019$). Thirty-one (54.4%) patients with active distal or left-sided ulcerative colitis received topical therapy, and 27.8% of patients suffering from severe inflammatory bowel disease did not receive oral or intravenous steroid therapy. Among the 51 patients for whom thiopurine was indicated, only 10 (19.6%) received immunomodulatory agents, and more than half of the 8 patients received a suboptimal dose of azathiopurine with no attempt to increase its dosage.

CONCLUSION: The quality of care for IBD patients can be further improved. A suboptimal dose of aminosalicylate is used in treatment of patients with CD, especially in those with small intestine involved only. Topical mesalazine is inadequately used in patients with distal or left-sided colitis. Oral or intravenous steroid therapy is not used in some patients with severe IBD. Use of immunomodulatory medication is limited. Larger prospective studies are needed to investigate the quality of care for patients with IBD to establish our own evidence-based guidelines.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Quality of care; Guidelines

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INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic disorder of the gastrointestinal tract. The prevalence of IBD is about 1.0/1000 in Western countries[1]. No large scale epidemiological investigation is available on the incidence and prevalence of IBD in China so far, but the diagnosed IBD in China has increased obviously in the past ten years[2].

A number of national and international practice guidelines for management of inflammatory bowel disease are available[3-5]. However, the essentials in a step-up treatment have not changed significantly. These practice guidelines are means of bringing evidence-based medicine into full play to improve IBD patient care. However, such

guidelines can improve the care of IBD patients only when they succeed in moving the actual clinical practice closer to the recommended practice.

The Sir Run Run Shaw Hospital is a teaching hospital affiliated to Zhejiang University, China. Together with 12 other large hospitals and numerous smaller district hospitals, it provides health care to 48 million people living in Zhejiang Province of East China. Our group has carried out a retrospective study to assess the demographic and clinical characteristics of IBD in Chinese patients[2]. This study was to investigate the impact of such guidelines and the quality of care for a hospital based cohort of patients with IBD from East China.

MATERIALS AND METHODS

Subjects

IBD patients admitted to Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, between June 2000 and June 2006, were consecutively enrolled in this study. The diagnosis of IBD was confirmed by the criteria established by Chinese Society of Gastroenterology[6] in 2000 and the guidelines issued by the Clinical Services Committee of the British Society of Gastroenterology (BSG)[3] in 2004. The patients not under the care of a gastroenterologist were excluded.

Data collection

A retrospective review was performed. Clinical data on demographic information, clinical characteristics of IBD patients, as well as endoscopic, radiologic, surgical and pathological records, confirmed diagnoses, duration and severity of disease, medical therapy, were collected from the inpatient and follow-up clinic visit records and input into an IBD database. Medical therapy included use of oral aminosalicylate, topical therapy, corticosteroid agents, and use of immunomodulatory agents at admission and outpatient clinic visit.

Statistical analysis

Variables were expressed as mean, while categorical data such as proportion of patients receiving particular therapies and dose of medications were expressed as percentage. Association of patient gender, age, location and severity of disease with suboptimal therapy was analyzed by χ^2 test. $P < 0.05$ was considered statistically significant. All statistical analyses were carried out with the SPSS v. 13.0.

RESULTS

Patient characteristics

A total of 177 eligible patients were evaluated in this study, including 71 patients with CD and 106 with UC. All the patients were the Han nationality Chinese. Disease activity was assessed using the Harvey-Bradshaw index (HBI) for CD and the Sutherland index for UC. Active disease was defined as a HBI value $\geqslant 5$ or a Sutherland index $\geqslant 3$. All the eligible patients had active disease at admission. Severity was broadly divided into mild, moderate or severe according to the Truelove & Witts' criteria for UC and the criteria established by Chinese Society of Gastroenterology for CD[6]. Mild CD was defined when the patient had no fever, abdominal tenderness, abdominal mass and obstruction, while severe CD was defined when the patient had persistent high fever, weight loss, nausea, vomiting, abdominal pain, diarrhea, anemia and complications. The characteristics of the patients and the information about the location of disease are given in Tables 1 and 2, respectively. Twenty-one gastroenterologists were involved in the treatment of the patients.

Table 1 Characteristics of patients with CD or UC

Characteristics	CD (n = 71)	UC (n = 106)
Sex (male/female)	41/30	56/50
Age (yr) [mean (range)]	35 (13-70)	45 (15-80)
Disease duration (yr) [mean (range)]	5.3 (0.5-14)	5.8 (0.5-23)
Smoker/non-smoker	17/54	26/80
Disease severity		
Mild	13	53
Moderate	38	37
Severe	20	16
Previous surgery	34	4

Table 2 Distribution of CD and UC

Disease location	n
Crohn's disease (71)	
Small intestine	37
Colon	6
Ileocolon	27
Upper gastrointestinal	1
Ulcerative colitis (106)	
Distal colitis	35
Left-sided colitis	22
Extensive colitis	47
Missing information	2

Medical therapy

Medical therapy for the patients including use of aminosalicylate, topical therapy, corticosteroids and immunomodulatory agents was analyzed, and the results are listed in Table 3.

Overall, 175 of 177 patients received oral aminosalisylate. Among them, 145 received mesalazine and 30 received sulphasalazine (SASP) (69/71 with CD, 106/106 with UC). The remaining 2 patients who did not receive oral aminosalicylate, had a history of adverse events, and therefore, received oral corticosteroids. A suboptimal dose of mesalazine or SASP was defined as a dose less than 4.0 g/d in patients with ileal or colonic CD or a dose less than 3.0 g/d in patients with UC. Among the 69 CD patients who received aminosalicylate, one had stomach involvement. Among the remaining 68 CD patients, 27 (39.7%) had a suboptimal dose. In contrast, all the 106 patients with UC were treated with an optimal dose. A further analysis was performed to elucidate factors associated with suboptimal doses of such medications. The variables were gender, age, location of disease, and severity of disease. It revealed no association other than a significantly higher incidence of

Table 3 Suboptimal dose used in treatment of CD and UC

Clinical parameters	Proportion (%)
Suboptimal dose of aminosalicylate	
Crohn's disease	27/68 (39.7)
Ulcerative colitis	0/106 (0.00)
Failure to use topical therapy	26/57 (45.6)
Failure to use corticosteroid	10/36 (27.8)
Failure to use immunomodulatory agents	41/51 (80.4)
Suboptimal dose of AZA	4/7 (57.1)

Table 4 Suboptimal dose of aminosalicylate and location of CD

Location of CD	Suboptimal dose (*n*)	Optimal dose (*n*)
Small intestine[a]	19	17
Colon	2	4
Ileocolon	6	20

[a]$P = 0.019$: A significantly higher incidence of suboptimal dose in CD patients with small intestine involved only.

suboptimal dose in CD patients with small intestine involved only ($P = 0.019$) (Table 4).

Of the 57 patients suffering from active distal (applies to colitis confined to the rectum or rectum and sigmoid colon) or left-sided (applies to colitis extending proximal to the sigmoid descending junction up to the splenic flexure) ulcerative colitis, 31 (54.4%) received topical therapy. Medications used in topical therapy included mesalazine ($n = 5$), corticosteroids ($n = 22$), antibiotics ($n = 12$) as well as traditional medications ($n = 15$).

Oral corticosteroid use was also assessed. Of the 177 patients (29/71 with CD, 31/106 with UC), 60 were treated with corticosteroids. No significantly different doses were applied in patients with mild to moderate disease or severe disease ($P = 0.391$) (mild to moderate disease: mean dose 35.4 mg/d, range 10-60 mg/d; severe disease: mean dose 38.7 mg/d, range 10-75 mg/d). Of the 36 patients with severe inflammatory bowel disease, 10 (27.8%) did not receive oral or intravenous steroid therapy.

Of the 177 patients, 51 were thiopurine indicated. Among these 51 patients, 23 were steroid-dependent (refers to a relapse when the steroid dose is reduced below 10 mg/d, or within 6 wk of stopping steroids), 4 were steroid-refractory (refers to active disease in spite of an adequate dose and duration of prednisolone > 20 mg/d for > 2 wk) and 24 had postoperative fistulation CD. However, only 10 (19.6%) patients received immunomodulatory agents (8 received oral azathioprine AZA, 2 received oral cyclosporine). The mean AZA dose used in the 8 patients was 1.52 mg/kg per day, ranging from 0.83 to 2.22 mg/kg per day. A suboptimal dose of AZA was defined as a dose less than 1.5 mg/kg in the absence of leukopenia or hepatotoxicity. Based on such criteria, one patient developed myelotoxicity and tolerated a lower dose. Of the remaining 7 patients receiving AZA, 4 (57.1%) received a suboptimal dose with no attempt to increase the dose. None of the patients was treated with methotrexate or inflixmab.

DISCUSSION

IBD, including UC and CD, is a complex disorder characterized by a wide range of variations in clinical practice. As the evidence is changing and rapidly growing, national and international societies are continuously updating the current diagnostic and treatment guidelines, for the sake of avoiding variations in clinical practice, and offering strategies for their effective implementation. Ultimately, the success of practice guidelines is determined by the physicians' acceptance, and perceived usefulness of the guidelines and their impact on patient care. Reddy *et al*[7] have investigated whether patients with IBD receive optimal therapy in America according to the practice guidelines. Since general information about the quality of care for patients with IBD in China is lacking, we conducted this study to examine the quality of care for IBD patients in our hospital.

Abundant evidence suggested that higher doses of aminosalicylates (4 g/day) are more effective for inducing remission in UC or CD. A dose-response trend for aminosalicylates was also observed[8]. Clinical improvement (but not necessarily remission) in ulcerative colitis is associated with the dose of aminosalicylate ($\geqslant$ 3 g/d)[8]. Although the role of aminosalicylate in the management of CD is less clear, it was reported that its higher doses ($\geqslant$ 4 g/d) are effective on active CD of the ileum and colon[9-11]. Compared with sulphasalazine (SASP), mesalazine is not significantly better, but has lower side effects[12]. In our hospital, SASP is still widely used to induce and maintain remission of mild to moderate UC and CD, because of its comparatively lower price. Besides, the incidence of UC is relatively higher and the adverse events of SASP seem lower in China than those in Western countries. The mainstay of treatment, however, has shifted towards the use of mesalazine. In our study, all the patients with UC received the optimal doses of aminosalicylate, while 39.7% patients with CD received its suboptimal doses. Furthermore, a significantly higher incidence of suboptimal dose was found in CD patients with small intestine involved only. This phenomenon may be explained by the relatively lower incidence of CD than that of UC in China and therefore the less understanding of this disease.

Topical therapy allows direct delivery of the active drugs to the site of inflammation, limiting systemic absorption and potential side effects. Topical therapy is the most effective approach, provided that the formulation reaches the disease. It also plays an important role in the maintenance of remission. Oral therapy combined with topical therapy is superior to a single agent[13-15]. Furthermore, meta-analysis has shown that aminosalicylate is significantly better than topical steroids in inducing clinical remission[16,17]. However, in our study, only 31 (54.4%) patients with distal or left-sided colitis received topical therapy, and topical mesalazine was only used in 5 patients. There is a tendency that topical therapy is thought to be less effective in clinical practice, quite reverse to the evidence. Actually, its lower efficacy may be due to the lack of preparations such as liquid enemas, foams, gels and suppositories, rather than due to the medication itself.

Corticosteroids can effectively induce remission of UC and CD. Oral or intravenous corticosteroids are recommended for severe UC and CD[3,4]. In our study, more than a quarter of patients suffering from severe inflammatory bowel disease did not receive oral or intravenous steroid therapy, possibly due to the lack of comprehensive evaluation of the patients' baseline states and reevaluation when exacerbations occurred. In addition, the patients' and even some physicians' fear of adverse effect played a part role. There is evidence that side effects of corticosteroid therapy are related to the dosage used and its duration. Short-term corticosteroid therapy is relatively safe.

Although corticosteroids are effective in inducing remission of CD and UC. More than 20% of patients may be steroid-refractory or become steroid dependent. Meta-analyses have shown that corticosteroids are not effective in maintaining medically or surgically induced remission of IBD. The frequency and severity of well recognized adverse effects also preclude their long term use. Immunomodulatory agents such as azathioprine (AZA) and mercaptopurine (MP) can induce and maintain remission of CD and UC and have steroid sparing effects in patients with steroid dependent or refractory IBD[21,22]. Although lower doses had been favored in early clinical trials, a meta-analysis demonstrated greater efficacy of higher doses[23,24]. In our study, the use of immunomodulatory agents was restricted to a minority of IBD patients (19.6%), which is distinctly less frequent compared with that in Western countries. Furthermore, more than half of the patients received a suboptimal dose of AZA with no attempt to increase its dosage. Biological agents such as infliximab have not been registered for the treatment of CD in our region, and their effect on CD in Han nationality Chinese needs to be further investigated.

The limited use of immulomodulatory medications may be due to the lack of evidence and limited experience with these drugs in Han nationality Chinese with IBD. Uncertainty regarding the risk for neutropenia deters some physicians from using AZA at effective doses and longer treatment with it. Experience abroad has shown that AZA can be safely used in treatment of CD and UC[25-27]. Recently, thiopurine methyltransferase (TPMT) and its genotype have been applied in predicting the myelotoxicity and reducing the incidence of leucopenia in Europe and North America[28-30].

In contrast, the data available on the management of IBD in China are rather limited. Guidelines published by the Chinese Society of Gastroenterology adhere mainly to the international ones. The lack of evidence holds back the use of these medications and therefore affects the quality of care for IBD patients. As the incidence of IBD has been increasing in China, it may be of interest to follow the temporal epidemiological trends and treatment policy for IBD in China.

Several factors limit the interpretation of our data. Firstly, we did not determine the duration or the frequency of visits. A longer period or more frequent contacts may provide more opportunities to achieve better care for IBD patients. Secondly, the patients' perspective and preference were not taken into account, which may affect the decision. Finally, we did not study the association of the reported quality of care with patient outcomes.

In conclusion, the quality of care for IBD patients can be improved. suboptimal doses of 5-ASA was used in treatment of patients with Crohn's disease, especially those with small intestine involved only and Topical mesalazine is inadequately used in treatment of patients with distal or left-sided colitis. Oral or intravenous steroid therapy is not used in treatment of certain patients with severe IBD. Use of Immunomodulatory agents is limited. Larger prospective studies are needed to investigate the quality of care for patients with IBD and to establish our own evidence-based guidelines.

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COMMENTS

Background

As the evidence is changing and rapidly growing, a number of national and international practice guidelines for management of inflammatory bowel disease (IBD) are available, for the sake of avoiding variations in clinical practice and offering strategies for their effective implementation. However, guidelines for IBD treatment can improve patients' care only when they succeed in moving actual clinical practice closer to recommended practice.

Research frontiers

Reddy *et al* have investigated the quality of care for patients with IBD in America, and suggested that patients often do not receive optimal treatment of IBD, despite the availability of practice guidelines.

Innovations and breakthroughs

Although the incidence of IBD has been increasing in China, general information about the quality of care for patients with IBD in China is lacking. We investigated the impact of guidelines and the quality of care for IBD patients from East China.

Applications

The results of this study suggest that the quality of care for IBD patients can be improved. Larger prospective studies are needed to investigate the quality of care for patients with IBD and to establish our own evidence-based guidelines.

Peer review

This is an interesting paper addressing an important issue in Chinese IBD patients. The paper is well written.

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RAPID COMMUNICATION

Polymorphism of thymidylate synthase gene associated with its protein expression in human colon cancer

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Abstract

AIM: To correlate the polymorphisms in the 5'-untranslated region with thymidylate synthase (TS) protein expression in Han Chinese colonic neoplasms.

METHODS: Adenocarcinoma samples were from 68 patients who received no treatment before surgery. Tandem repeat length of TS gene was determined by PCR amplification of genomic DNA. Intratumoral TS protein expression was studied immunohistochemically in corresponding sections from paraffin-embedded primary foci. Immunoreactivity was semiquantitatively evaluated by immunoreactivity score (IRS).

RESULTS: Double-(2R) and triple-repeated (3R) sequences of the TS gene were found in the cancer tissues. Three genotypes of TS were found: 2R/2R (n = 6), 2R/3R (n = 22) and 3R/3R (n = 40). Patients who were homozygous for triple-repeated (3R/3R) sequences showed significantly higher IRS of TS than patients who were homozygous for double-repeated (2R/2R) sequences or heterozygous patients (2R/3R): 5.73 ± 3.25 *vs* 2.17 ± 1.47 or 3.77 ± 2.64, P = 0.008 or P = 0.015. But no statistical significance of IRS in cancer tissues was observed between 2R/3R genotype and 2R/2R genotype.

CONCLUSION: There is a relationship between TS genotype and TS protein expression in clinical specimens. The data might offer an advantage for selection of Chinese cancer patients to receive fluoropyrimidines treatment.

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Key words: Colonic neoplasms; Thymidylate synthase; Polymorphism Genetic; Polymerase chain reaction

INTRODUCTION

Thymidylate synthase (TS) catalyses deoxyuridine-5' -monophosphate (dUMP) to 2'-deoxythymydine-5' -monophosphate (dTMP). This is the only *de novo* source of thymidylate, an essential precursor of DNA biosynthesis[1,2]. The critical role of TS in nucleotide metabolism has made it an important target for cancer chemotherapeutic drugs such as 5-fluorouracil (5-FU), whose mechanism of action is primarily mediated through competitive TS inhibition[3-5].

Several studies have suggested that intratumoral TS protein expression before the chemoradiation treatment was inversely correlated with response to 5-FU chemotherapy. Patients with low TS levels have better clinical outcome than those with high TS levels[6-13].

TS expression is a tightly controlled process regulated by the proliferative status of the cell. Special 28-base pair-sized, unique tandem repeated sequences have been descried in the 5-untranslated region of TS gene[14-16], and this polymorphism is identified to be various ethnically[17-20]. There are three predominant genotypes of TS: (1) homozygous with two tandem repeats (2R/2R); (2) homozygous with three tandem repeats (3R/3R); (3) heterozygous with both alleles (2R/3R). It was reported that TS genes with the triple repeats have higher expression activity than those with double repeats *in vitro* and *in vivo*[15,21,22]. In this study, we investigated the association of TS genotype with its protein expression in clinical specimens of colon adenocarcinoma.

MATERIALS AND METHODS

Materials

Sixty-eight tumor samples obtained by surgical resection in patients (Han Chinese) who received no treatment before surgery were studied. The tumor tissue (1 g) from primary foci was obtained and frozen immediately in liquid

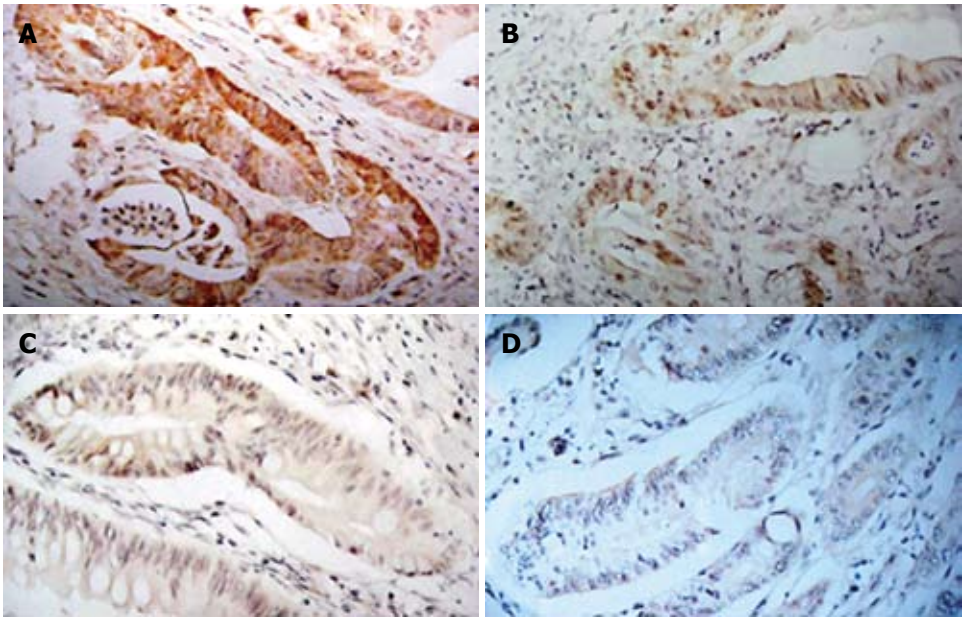


Figure 1 Immunohistochemical detection of TS staining intensity on paraffin sections of colorectal carcinoma. **A**: Strong; **B**: moderate; **C**: Weak; **D**: Absent (× 200).

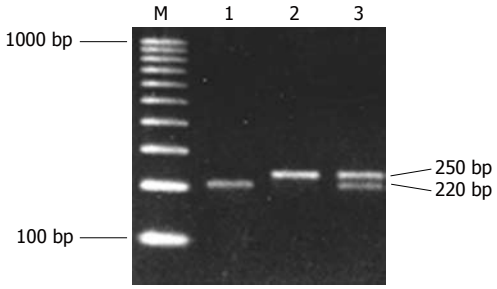


Figure 2 Electrophoresis of amplified products of patients with different TS TR genotypes: 220 bp (2R/2R), 248 bp (3R/3R) or both (2R/3R). Lane 1: 220 bp (2R/2R); Lane 2: 248 bp (3R/3R); Lane 3: 220 bp and 248 bp (2R/3R); M: molecular weight marker, a 100 bp ladder.

nitrogen until molecular analysis. The paraffin-embedded, formalin-fixed specimens from corresponding tumor were analyzed by immunohistochemistry.

Immunohistochemical evaluation

The monoclonal antibody TS 106[23] was used to detect TS. The standard Streptavidin-Peroxidase complex (Maixin-Bio, Fuzhou) technique was used. Immunoreactivity was evaluated semiquantitatively: labeling intensity (absent, weak, moderate, strong; Figure 1) × percentage of cells in each of the four intensity categories (0%-1%, 1%-25%, 26%-50%, 51%-75% and 76%-100%). In order to analyze the individual immunostaining result, integer values were assigned to the intensity scores (0-3) and the proportion of cells stained (0-4). These values were multiplied to provide a single integrated immunoreactivity score (IRS) for TS. All slides were randomly allocated for independent assessment by two observers, blinded to marker status. The agreement of IRS reached by two independent observers was > 90%. If there was disagreement, IRS was determined by consensus.

DNA extraction

DNA was extracted from the samples which were stored immediately at -80℃. After overnight incubation in 100 ng/mL Proteinase K (Promega, Southampton, UK) in 50 mmol/L TrisHCL (pH 7.5) and $CaCl_2$ 5 mmol/L solution at 37℃, DNA was extracted by the phenol-chloroform method.

Polymerase chain reaction (PCR)

The sequences of the primers used were TS12 5'-GTGG CTCCTGCGTTTCCCCC-3' (sense) and TS18 5'-GCTCC GAGCCGGCCACAGGCATGGCGCGG-3' (antisense). PCR analysis was performed in a total volume of 25 μL: 1.5 μL DNA, 1 μL (10 μmol/L) antisense and sense mixed primer, 0.25 μL (5 U/μL) Taq DNA polymerase (TaKaRa LA Taq LotE5801-7, code RR002A), 12.5 μL of 2 × GC buffer, 4 μL of 2.5 mmol/L mix of dNTP, and 5.75 μL DDW on Gene Amp PCR System 9700 (Perkin Elmer, Foster city, Norwalk, CT). All PCR reagents were obtained from TaKaRa Biomedicals (Otsu, Shiga, Japan). After 30 cycles of amplification (denaturation at 94℃ for 30 s, annealing at 70℃ for 30 s, extension at 72℃ for 30 s), the amplified products were electrophoresed in 2% agarose gel. Products of 220 bp (2R/2R), 248 bp (3R/3R) or both of these products (2R/3R) were obtained based on the TS TR genotype (Figure 2).

Cloning and sequencing of PCR products

PCR products were subcloned using the pGEM-T Vector System (Promega, Madison, WI). Subsequently the cloned PCR products were sequenced with a Thermo Sequenase Cy5.5 Terminator Sequencing kit (Amersham-Pharmacia, Piscataway, NJ). For confirmation of the sequence, at least five clones were analyzed.

RESULTS

TS polymorphisms: distribution among samples

In the studied samples, the following frequencies of TS genotypes were detected: 2R/2R in 6 patients (9%), 3R/3R in 40 patients (59%), and 2R/3R in 22 patients (32%) (Figure 3). Tandem repeated sequences in TS DNA:
2R: CCGCGCCACTTGGCCTGCCTCCGTCCCG--

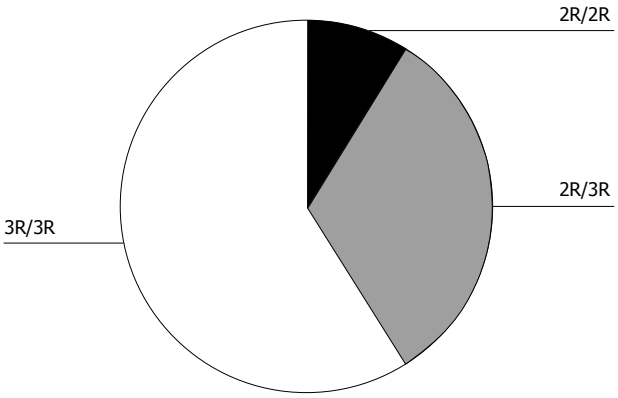


Figure 3 Distribution of TS polymorphisms.

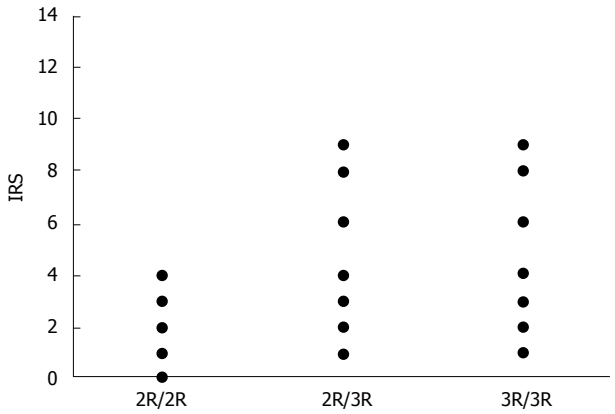


Figure 4 Association of TS genotype with IRS of TS. IRS: Immunoreactivity score; TS: Thymidylate synthase.

CCGCGCCACTTCGCCTGCCTCCGTCCCC
3R: CCGCGCCACTTGGCCTGCCTCCGTCCCG--CCGCGCCACTTGGCCTGCCTCCGTCCCG--CCGCGCCACTTCGCCTGCCTCCGTCCCC

TS genotype and IRS of TS

Cancer tissues with 3R/3R genotype showed significantly higher IRS of TS than those with 2R/3R genotype (5.73 ± 3.25 *vs* 3.77 ± 2.64, $P = 0.015$). Similarly, IRS of TS in 3R/3R genotype tissues was higher than that in 2R/2R genotype (5.73 ± 3.25 *vs* 2.17 ± 1.47, $P = 0.008$). But there was no statistical significance of IRS in cancer tissues between 2R/3R genotype and 2R/2R genotype (Figure 4).

DISCUSSION

There is an increasing need for defining new factors that may be used to effectively forecast the clinical response of colon cancer to a particular chemotherapy. Measurements of TS in colon cancer have been shown to be of interest because of the possible role of this enzyme in the clinical prognostic value to 5-FU-based chemotherapy[6,8,9,13,24-28]. However, the classical assay for TS-activity determination (high-performance liquid chromatography with output monitored by radioactive flow detector) is tedious and expensive. Edler *et al*[29] and Van Triest *et al*[30] compared the TS enzyme activity of lysates from frozen-stored colorectal cancer specimens with TS staining intensity using the monoclonal antibody TS106, and found a statistically significant correlation between the peak enzyme activity values and the TS staining intensity of respective colorectal cancer samples. IRS of TS in the paraffin-embedded specimen, a semiquantitative analysis of the individual immunostaining results, seems to be a good alternative to evaluate the intratumoral TS enzyme activity. Therefore, our observation of the link between TS genotype and IRS of TS suggested that TS genotype can be a genetic factor which can be used to predict the patient's response to 5-FU-based chemotherapy.

The present investigation demonstrated that there was a correlation between TS genotype and IRS of TS. Patients with the 3R/3R genotype showed significantly higher intratumoral IRS of TS compared to those with the 2R/3R genotype. Similarly, intratumoral IRS of TS in 3R/3R genotype tissues was higher than that in 2R/2R genotype. But no statistical significance of IRS was observed between 2R/3R genotype and 2R/2R genotype. Our results support the previous observation that the expression activity of the TS gene with the triple repeats is higher than that with the double repeats in a transient expression assay[15]. One of the mechanisms by which the repeat length of the TS gene influences its protein expression is that the three-repeat sequence has greater translation efficiency than that with two-repeat sequence[22].

Ethnic variations of the TS genotype have been reported in the literature[17-20]. The frequency for homozygous triple repeats arranged from 30% to 54% in the Caucasian population. In the Latino population, TS genotype distribution has been reported to be 16% in the 2R/2R group, 42% in the 2R/3R group, and 42% in the 3R/3R group. In the present study with Han population, the allelic frequency of triple repeats was 75%. According to Luo *et al*[31], the frequency of allele 3 varied from 62% to 95% in different Chinese populations.

In conclusion, our results support the previous *in vitro* expression study and suggest the association between TS genotype and TS protein expression in clinical specimens. These data are important for pharmacogenomics analysis in 5-FU-based chemotherapy. Prospective, large-scale trials are needed to confirm that the TS polymorphism is a useful molecular tool for optimizing drug therapy for the cancer patients.

COMMENTS

Background

As a pyrimidine analog, 5-fluorouracil (5-FU) exerts its antitumor effect through competitive thymidylate synthase (TS) inhibition. It was noted that the outcomes differed greatly among patients who received 5-FU-based adjuvant chemotherapy, whose efficacy seem to be affected by the intratumoral TS level. Polymorphism in 5'-untranslated region of TS gene influences the efficacy of TS expression, and this polymorphism was identified to be various ethnically. In this experiment, association of TS genotype with its protein expression in clinical specimens of Han Chinese with colon adenocarcinoma was observed.

Research frontiers

To associate the presence of the tandem repeat sequences within the 5'UTR of the TS gene with intratumoral TS expression, the TS expression was assessed by the immunoreactivity score (IRS). However, there has been no report about the relationship between TS gene polymorphisms and TS protein expression in Han Chinese patients.

Innovations and breakthroughs

Immunoreactivity score was applied to evaluate the TS expression level semiquantitatively to improve the study efficiency.

Applications

Prospective, large-scale trials are needed to confirm our results and to establish the advantages of the TS polymorphism as a useful molecular tool for optimizing fluoropyrimidine-based therapy in Han Chinese cancer patients.

Terminology

Thymidylate synthase (TS) is the key enzyme that catalyzes methylation of fluorodeoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is an important step in the process of DNA synthesis.

Peer review

This study associated the presence of the 3R/3R tandem repeat sequence within the 5'UTR of the thymidylate synthase gene with increased tumor TS expression as assessed by IHC. The study was conducted in a cohort of 68 Han Chinese patients with colon cancer. It was relatively well performed and appropriate conclusions have been drawn.

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RAPID COMMUNICATION

Significance of antineutrophil cytoplasmic antibody in adult patients with Henoch-Schönlein purpura presenting mainly with gastrointestinal symptoms

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Abstract

AIM: To test the clinical significance of antineutrophil cytoplasmic antibody (ANCA) in evaluation of adult Henoch-Schönlein purpura (HSP) patients presenting mainly with abdominal symptoms.

METHODS: Twenty-eight consecutive HSP patients who presented predominantly with abdominal symptoms were enrolled in this study. Control subjects included 27 age- and sex-matched patients with peptic ulcer disease, colon cancer, acute gastroenteritis, irritable bowel syndrome and colonic polyps. ANCA was measured by indirect immunofluorescence (IIF) in all patients, and follow-up ELISA was performed in patients with positive IIF tests.

RESULTS: ANCA was detected in 9 HSP patients by IIF (2 were positive for c-ANCA and 7 were positive for p-ANCA). No ANCA was found in the control group. The sensitivity and specificity of a positive ANCA test (either c- or p-ANCA) were 32.1% and 100% respectively. Only one out of the 9 patients with positive ANCA by IIF had positive ANCA by ELISA and the antigen was myeloperoxidase (MPO). The patients positive for ANCA had higher HSP clinical scores, and were more likely to have renal function impairment. Patients with late purpura development were also associated with more severe clinical manifestations.

CONCLUSION: A positive ANCA test is associated with more severe symptoms in HSP. After inflammatory bowel disease is excluded, a positive ANCA test provides a clue to the diagnosis of HSP presenting predominantly with abdominal symptoms.

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Key words: Henoch-Schönlein purpura; Antineutrophil cytoplasmic antibody; Diagnosis; Indirect immunofluorescence

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INTRODUCTION

Abdominal pain, diarrhea, hematochezia are the common symptoms seen in clinical gastroenterology. The underlying causes may not always be clear. Henoch-Schönlein purpura (HSP) is one of the uncommon causes and can be a challenging clinical problem[1]. Among the presenting symptoms, gastrointestinal (GI) complains occur in 50% to 85% of patients[2]. Some HSP patients may develop serious GI complications, such as intussusception, obstruction and perforation, which may require surgical intervention on rare occasions[3-5]. Early diagnosis of HSP presenting mainly with acute abdominal symptoms in adults remains a challenge to both gastroenterologists and general surgeons, particularly if the abdominal symptoms precede the typical skin rash. Although HSP is more common in children, it has also been documented in adults[6]. In comparison to children, adult patients have more atypical symptoms and a poorer prognosis and need more intensive care[7]. Although lots of efforts have been made, the mortality in adults with HSP is still higher than that in children. Doctors are eager to find a more effective therapy for the disease.

Since antineutrophil cytoplasmic antibody (ANCA) was reported in 1982 by Bavis, many studies have shown that ANCA is a useful tool for the diagnosis of systemic

vasculitis, especially for the diagnosis and follow-up of microscopic polyangiitis and Wegener's granulomatosis[8,9]. It was reported that ANCA is also detectable in patients with HSP. However, the prevalence of HSP remains controversial, ranging 0%-79%[10-13]. It has been shown that the positive ANCA rate is as high as 82.3%[14], suggesting that ANCA can be used to confirm the diagnosis of HSP in children. In addition, some reports indicate that ANCA is also related to the severity of autoimmunological diseases, such as Wegener's granulomatosis[15], systemic vasculitis[16,17]. However, the relationship between ANCA and the severity of HSP has not been extensively investigated.

Till now, most studies were confined to children, only a few studies have been done in adults[12,13] and no reports on adult HSP patients presenting mainly with gastrointestinal symptoms are available. The aim of this study was to evaluate the clinical significance of ANCA in adult HSP patients who presented mainly with gastrointestinal symptoms and its role in the diagnosis of HSP and its relationship to the disease severity.

MATERIALS AND METHODS

Patient selection

From February 2003 to March 2006, all adult HSP patients (age > 20 years) with prominent gastrointestinal symptoms, admitted to West China Hospital of Sichuan University, were enrolled in the study. All the patients fulfilled the HSP diagnostic criteria[18]. During the same period, non-HSP inpatients with similar gastrointestinal symptoms served as the control group. Only patients without preexisting medical comorbidity were included in this study. Patients with known inflammatory bowel disease or autoimmune diseases were excluded from the study.

ANCA detection by indirect immunofluorescence (IIF)

The method is commercially established and provided by EUROIMMU Company (Germany). Briefly, serum samples were diluted at 1:3.2 in a phosphate- buffered solution (PBS), then 25 μL was applied to slides, and incubated at room temperature for 30 min. After the slides were washed with PBS, 20 μL FITC-labeled affinity-purified goat anti-human IgG (EUROIMMU Company, Germany) was applied to the slides and incubated at room temperature for 30 min. Fluorescence microscopy was performed and the results were interpreted as negative for lack of fluorescence and positive for the presence of moderate or intense fluorescence.

ANCA detection by ELISA for PR-3, MPO, EL, Cath-G, LF and BPI

The ELISA kit from EUROIMMU Company (Germany) was used. Briefly, 100 μL of patient serum diluted at 1:100, positive and negative controls were put in 96-well plates (coated with PR-3, MPO, EL, Cath-G, LF or BPI) and incubated at room temperature for 30 min. After washed with PBS, 100 μL enzyme-labeled rabbit antihuman IgG (EUROIMMU Company, Germany) was added to the wells and incubated for 30 min. After washing, 100 μL enzyme substrate (TMB/H_2O_2) was added to the wells, and incubated for 15 min. The reaction was then terminated by adding 100 μL sulfuric acid (0.5 mol/L). The absorbance was read at 450 nm with an automated plate reader. The result was expressed as (serum sample OD-OD of negative control)/[(C-blank OD) × 0.2)]. A value < 0.1 was regarded as negative and ⩾ 0.1 as positive.

Table 1 Clinical scores for HSP

Symptom	Score
Purpura	
below the umbilicus	1^+
above the umbilicus	2^+
Abdominal pain	
Stool occult blood < 2^+	1^+
Stool occult blood > 2^+	2^+
Joint pain	1^+
Renal involvement	
Urinary occult blood 1^+	1^+
Urinary occult blood 2^+	2^+
Urinary occult blood 3^+	3^+

Other biochemical examinations

Twenty four-hour urinary protein, urinalysis, kidney function and antinuclear antibody (ANA) test and rheumatoid factor (RF) were performed in the clinical laboratory of the hospital.

HSP clinical score

To stratify patients according to the disease severity, the clinical scoring system of was used as previously described Table 1[19].

Statistical analysis

Results were given as mean ± SD and percentage. For statistical analysis, Student *t* test (for age, clinical scores, blood creatine, 24 h urinary protein) and χ^2 test (for sex and clinical symptom percentages) were used. $P < 0.05$ was considered statistically significant.

RESULTS

Twenty-eight patients with a final diagnosis of HSP who presented mainly with gastrointestinal symptoms were included in the study. All HSP patients eventually developed typical purpura lesions. The average clinical score was 5.32 ± 0.92. Twenty-seven patients with peptic ulcer ($n = 10$), colon cancer ($n = 1$), acute gastroenteritis ($n = 6$), irritable bowel syndrome ($n = 6$), and colonic polyps ($n = 4$) served as controls. Age and sex distribution were comparable between the two groups ($P > 0.05$). The demographics, symptom percentages in both groups are listed in Table 2.

All the patients were negative for ANA. Only one HSP patient was found to have a positive RF test. Of the 23 patients who could recall the occurring time of their symptoms, 5 complained of GI symptoms preceding purpura while 18 complained of purpura preceding the GI symptoms.

ANCA was detected in 9 HSP patients. Of them, 2 had

Table 2 Comparison of demographic data between HSP patients and controls

	HSP	Control	*P* value
n	28	27	NS[1]
Gender (male/female)	15/13	11/16	NS
Age (yr) (mean ± SD)	29.8 ± 8.1	30.1 ± 8.6	NS
Age range (yr)	20-46	22-52	
Symptoms			
Abdominal pain (%)	53.6	59.8	NS
Hematochezia (%)	46.4	51.2	NS
Diarrhea (%)	21.4	32.1	NS
Vomiting (%)	10.7	14.8	NS

[1]NS denotes no significance.

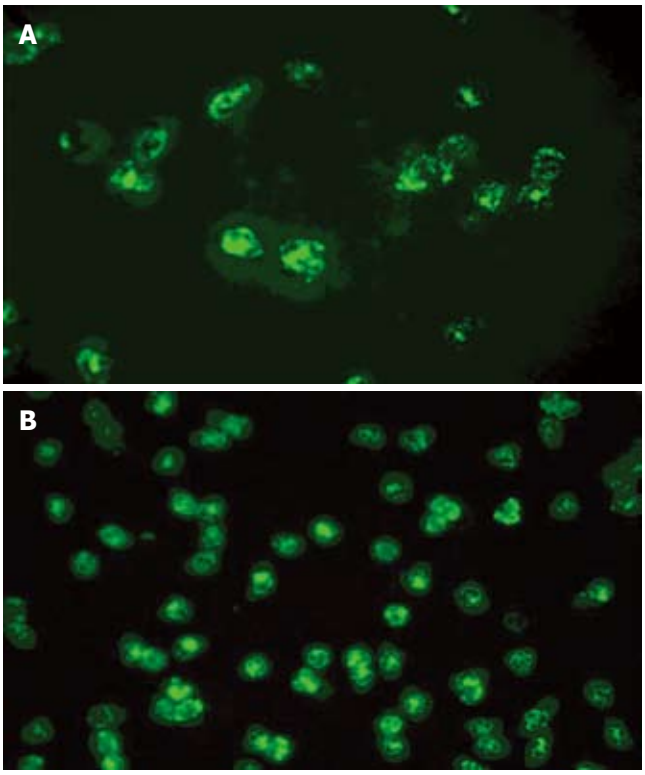


Figure 1 Strong cytoplasmic (**A**) and nuclear (**B**) staining patterns of ANCA (C-ANCA and P-ANCA) detected by indirect IIF. Serum samples from patients were diluted and incubated with primary antibody, then with FITC-labeled affinity-purified goat anti-human IgG. The results were observed under fluorescence microscope.

C-ANCA (Figure 1A) and 7 had P-ANCA (Figure 1B). No ANCA was detected in the controls (Table 3). The sensitivity and specificity of positive ANCA were 32.1% and 100% respectively. ELISA was performed for the 9 patients with positive ANCA detected by indirect IIF. Only one patient was positive for ANCA and the antigen was myeloperoxidase (MPO).

HSP patients who were positive for ANCA had higher clinical scores (6.79 ± 0.72 *vs* 4.12 ± 0.63, $P = 0.02$), and a higher rate for renal involvement as indicated by serum creatine level and 24 h urinary protein at the time of diagnosis (Table 4). Baseline kidney function tests were not available for most patients. However, given the lack of preexisting medical conditions, HSP was the most likely culprit for renal impairment in these patients.

Table 3 ANCA detected by IIF in HSP patients and controls

	+ANCA by IIF	- ANCA by IIF
HSP	9	19
control	0	27

Table 4 Blood creatine and 24 h urinary protein in HSP patients (mean ± SD)

	Blood creatine (μmol/L)	24 h urinary protein (g/L)
ANCA (+)	320 ± 22 (3.64 ± 0.25 mg/dL)	1.78 ± 0.12
ANCA (-)	104 ± 15[b] (1.19 ± 0.16 mg/dL)	0.78 ± 0.32[c]

[b]$P = 0.01$, [c]$P = 0.03$.

Table 5 Characteristics of the two groups

Symptom sequence	Positive ANCA (%)	HSP score	Abdominal pain (%)	Diarrhea (%)	Hematochezia (%)
Purpura before abdominal symptoms	33.3	5.02 ± 1.23	55.6	22.2	38.9
Purpura after abdominal symptoms	40.0	6.23 ± 1.08	60.0	40.0	60.0

HSP clinical scores and ANCA positive rates in patients with early and late purpura were also analyzed (Table 5). The latter was associated with a higher clinical score, but the GI symptoms and ANCA positive rate were similar in the two groups.

Typical case presentation

Case 1: A 28-year-old male was admitted for vomiting and diarrhea for 5 d and oliguria for 1 d. Physical examination on admission showed mild tenderness in the mid upper abdomen. Stool analysis showed white blood cells (+) and red blood cells (+). Renal function test showed moderately elevated blood BUN and creatine. Urinary analysis showed occult blood (++). "Acute gastroenteritis and acute renal function failure" were the initial diagnosis on admission. Infusion of 2 liters of intravenous fluid did not result in any clinical improvement. Serum ANCA test was positive. Three days later, typical purpura appeared on his lower extremities and HSP was thus diagnosed. The symptoms disappeared after one-month intravenous cyclosporine therapy.

Case 2: A 26-year-old male was admitted for abdominal pain and hematochezia for 4 d, and hematuria for 1 d. Physical examination was normal. Blood examination showed moderate anemia. Urinalysis showed red blood cells (++). Renal ultrasound was normal. An upper endoscopy showed edema and congestion in the pyloric region (Figure 2A). Colonoscopy showed several petechiae scattering throughout the colon (Figure 2B). ANCA test was positive. Four days later, typical purpura appeared in his hips and lower extremities. All symptoms disappeared three weeks after treatment with prednisone.

DISCUSSION

HSP is an immune-complex mediated vasculitis, affecting small vessels in skin, gut and glomeruli[20] and is characterized by polymorphonuclear leukocytic infiltration of the vessel wall. Mucosal lesions of HSP can occur anywhere in the GI tract, and the small intestine is unfortunately the most commonly affected[21,22]. In 10%-15% HSP patients, gastrointestinal symptoms precede the cutaneous lesions[23]. The GI manifestation in these patients is usually severe and sometimes requires laparotomy. Our study also showed that the patients with GI symptoms preceding the purpura had higher clinical scores, but unfortunately their GI symptoms were nonspecific.

ANCA is an auto-antibody existing in peripheral blood, and has a high specificity for and sensitivity to certain vasculitis. Saulsbury tested ANCA in 29 HSP patients and none was positive[10], suggesting that ANCA is not a useful diagnostic tool for HSP. Renato and Robson *et al*[11,12] have also found a low prevalence of ANCA in HSP, indicating that ANCA is not related to HSP. On the contrary, it was reported the prevalence (79%) of ANCA is high in HSP patients[13,24]. Faith *et al*[14] also showed that IgA ANCA is 82.3% in children with HSP, demonstrating that ANCA is a useful test to confirm the diagnosis of HSP in children. In our study, the specificity and sensitivity of ANCA were 100% and 32.1%, respectively. Due to its high specificity, a positive test result can be very helpful in making the early diagnosis of HSP before cutaneous lesions occur. However, our study excluded ulcerative colitis (UC) patients. According to recent studies, ANCA is recorded in 50%-70% of patients with UC[25]. Because UC has its own characteristic clinical manifestations, especially specific colonoscopic manifestation, it is very easy for GI doctors to differentiate UC from HSP.

The reasons why the reported results are different may be as follows. (1) IIF is a crude test method its results may be affected by many factors such as RF and fibronectin, and interfered with the different process and conditions of the study. In our study, RF was found only 1 patient, suggesting that it is not the reason why ANCA test was positive in our study. (2) Specific antigens are insufficient. The main target antigens of ANCA lay in the azurophil granules of neutrophils. Only five specific antigens have been identified so far for ANCA, including proteinase 3 (PR-3), myeloperoxidase (MPO), leukocyte elastase (EL), cathepsin G (CG), lactoferrin (LF)[26]. In our study, we tested the binding of the five antigens to ANCA by ELISA. The result showed that only one HSP serum sample had reactivity against anti-MPO, which is in agreement with previous reports, indicating that the five antigens are not specific antigens for ANCA. However, little is known about the specific antigens for ANCA. Ronda *et al*[13] analyzed the target antigen by Western blot using four strongest ANCA positive serum samples and found that a 51-kDa protein can be recognized in the neutrophil extract, indicating that the target antigens of ANCA may be different in patients with HSP and vasculitis. Discovery of more specific antigens for ANCA is needed to improve its value for clinical utilization. (3) It also may be related to the selection bias of cases. Some studies showed that different expressions of HLA are interfered with the expression of ANCA.

Our results show that a positive ANCA test was associated with a higher HSP score and a higher rate for renal dysfunction and urinary abnormality, which is consistent with the data reported by Shaw and Esnault[27,28], who found that patients with worse kidney function and higher urinary protein level have a higher ANCA titer, showing that ANCA is related to the activity of HSP. These findings suggest that ANCA may take part in the pathogenesis of HSP. The presence of ANCA in the sera of patients may exert both stimulatory and inhibitory effects on neutrophil and monocyte functions. It can stimulate the oxidative burst and degranulation, leading to the release of reactive oxygen intermediates. Enzymes from granules may directly damage the target tissues, inhibit the microbicidal activity of polymorphonuclears, thus aggravating the neutrophil/macrophage dysfunction by inadequate elimination of the infectious agents[29]. This may lead to the Th1/Th2 lymphocyte imbalance, leading to tissue damage and perpetuation of chronic inflammation. In addition, ANCA may influence the process of physiologic cell death. It was reported that ANCA can induce apoptosis of neutrophils, phagocytosis of apoptotic neutrophils opsonized by ANCA induces oxidative metabolism of monocytes, and defective apoptosis of neutrophils may thus exacerbate the tissue damage[30].

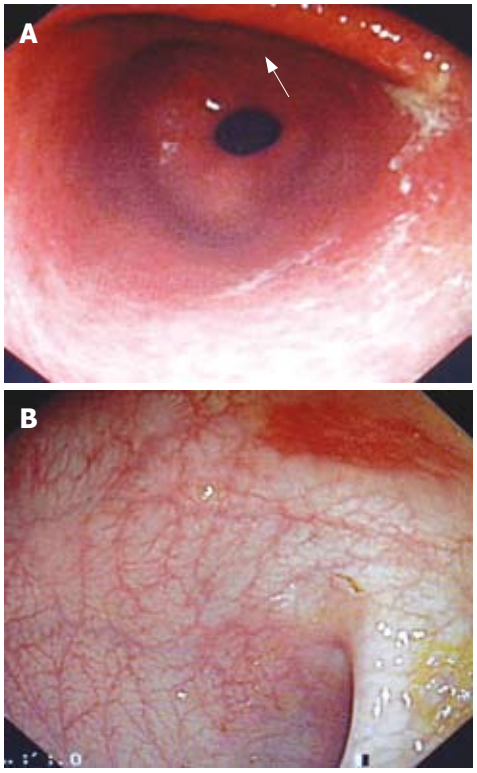


Figure 2 Gastroendoscopy showing edema and congestion in the pyloric region (**A**) and several petechiae scattering throughout the colon (**B**).

In conclusion, after exclusion of UC, a positive ANCA test in patients presenting mainly with GI symptoms can provide a clue to the diagnosis of HSP. Patients with a positive ANCA develop more severe symptoms and are more likely to have their kidneys involved. HSP patients with positive ANCA initially presenting with only GI symptoms should receive a more aggressive therapy. Since our sample was small, further studies are needed to find the exact antigens for ANCA in HSP patients.

COMMENTS

Background

Gastrointestinal symptoms are the common symptoms of Henoch-Schönlein purpura (HSP). Early diagnosis of HSP with gastrointestinal symptoms is difficult and some patients even undergo unnecessary laparotomy. Improving the early diagnosis of HSP still remains a challenge to both gastroenterologists and general surgeons.

Research frontiers

Antineutrophil cytoplasmic antibody (ANCA) is a useful tool for the diagnosis of systemic vasculitis. Recent studies indicate that ANCA is also detected in patients with HSP. However, its prevalence remains controversial. In addition, it was reported that ANCA is also related to the severity of many autoimmunological diseases, but the relationship between ANCA and the severity of HSP has not been extensively investigated.

Innovations and breakthrough

Most studies on HSP have been confined to children and no reports are available on adult HSP patients presenting mainly with gastrointestinal symptoms. In the present study, the authors found that ANCA positive test had a high specificity in HSP patients with gastrointestinal symptoms and HSP patients with ANCA positive had a higher clinical score.

Applications

ANCA may be a useful marker for the diagnosis of HSP with gastrointestinal symptoms.

Peer review

This paper investigated the significance of ANCA in HSP patients presenting with gastrointestinal symptoms. The authors concluded that a positive ANCA test could diagnose HSP in adult patients. HSP patients with positive ANCA have more serious symptoms.

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RAPID COMMUNICATION

Anticancer activity of genistein on implanted tumor of human SG7901 cells in nude mice

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Abstract

AIM: To investigate genistein-induced apoptosis of implanted tumors of SG7901 cells in nude mice, and the relationship between this apoptosis and expression of Bcl-2 and Bax.

METHODS: Establishing a transplanted tumor model by injecting human SG7901 cells into subcutaneous tissue of nude mice. Genistein (0.5, 1 and 1.5 mg/kg) was directly injected adjacent to the tumor, six times at 2-d intervals. Then, changes in tumor volume were measured continuously and tumor inhibition rate of each group was calculated. We observed the morphological alterations by transmission electron microscopy (TEM), measured the apoptotic rate by the TUNEL staining method, and detected the expression of apoptosis-regulated gene Bcl-2 and bax by immunohistochemical staining and RT-PCR.

RESULTS: Genistein 0.5, 1 and 1.5 mg/kg significantly inhibited carcinoma growth when it was injected near the tumor by 10.8%, 29.9% and 39.6%, respectively. Genistein induced implanted tumor cells to undergo apoptosis, with apoptotic characteristics seen by TEM. The apoptosis index was increased progressively with increasing genistein dose (28.9% ± 1.2%, 33.8% ± 1.6% and 37.7% ± 1.2%). The positive rate of Bcl-2 protein was decreased progressively (11.9% ± 0.9%, 5.9% ± 0.7% and 4.2% ± 0.6%), and the positive rate of bax protein was increased progressively (0.9% ± 1.7%, 24.9% ± 0.8% and 29.6% ± 1.7%) by immunohistochemical staining, with increasing dose of genistein. The density of Bcl-2 mRNA decreased progressively and the density of bax mRNA increased progressively with elongation of time by RT-PCR.

CONCLUSION: Genistein was able to induce apoptosis of transplanted tumor cells. This apoptosis may be mediated by down-regulation of the apoptosis-regulated gene Bcl-2 and up-regulation of apoptosis-regulated gene bax.

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Key words: Genistein; Gastric carcinoma; Nude mice; Apoptosis

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INTRODUCTION

The Bcl-2 family plays a crucial role in the control of apoptosis. The family includes a number of proteins which have homologous amino acid sequences, including anti-apoptotic members such as Bcl-2 and Bcl-xl, as well as pro-apoptotic members including Bax and Bad[1-4]. In *in vitro* experiments, overexpression of Bcl-2 has been shown to inhibit apoptosis[5-9], but overexpression of Bax has been shown to promote apoptosis[10-14].

Genistein has estrogenic properties in receptor binding assays[15,16], cell culture[17,18], and uterine weight assays[19-21]. Genistein inhibits microsomal lipid peroxidation[22] and angiogenesis[23]. Genistein exhibits antioxidant properties[24-26] and has been reported to induce differentiation of numerous cell types[27-29]. Moreover, a recent report has shown that genistein is a potent cancer chemopreventive agent[30-32]. The anti-tumor activity of genistein might be related to the induction of apoptosis of tumor cells but the precise mechanism of its antitumor activity is not well understood.

This study investigated genistein-induced apoptosis of implanted tumors of SG7901 cells in nude mice, the relationship between this apoptosis and expression of Bcl-2 and bax *in vivo*, and the theoretical and methodological basis of the clinical application of genistein.

MATERIALS AND METHODS

Materials

Genistein was obtained from Sigma and dissolved in dimethylsulfoxide (DMSO). In situ cell detection kit, anti-Bcl-2 monoclonal antibody and anti-Bax monoclonal antibody were purchased from Beijing Zhongshan Biotechnology. Female Balb/C nude mice (4 wk old, 16-18 g) were obtained from Chinese Academy of Medical Science.

Cell culture

Fresh samples from a patient with low-differentiation gastric cancer were obtained in the operating room. A single-cell suspension of tumor cells with a concentration of 5×10^5/mL was prepared for seeding. SG7901 cells were artificially purified after culture by pancreatic proteinase.

Tumor implantation into nude mice

A transplanted tumor model was established by injecting 1×10^9 human SG7901 cells/L into the subcutaneous tissue of nude mice. After 10 d, 25 nude mice were randomly divided into five groups, and 0.2 mL normal saline solution, 1.5 mg/kg DMSO, or 0.5, 1 or 1.5 mg/kg genistein was directly injected adjacent to the tumor, six times at 2-d intervals. Changes in tumor volume [$V = (\pi/6) \times abc$] were measured at 11 d after drug treatment and the tumor inhibition rate of each group was calculated.

$$\text{Inhibitory rate (IR) of tumor growth} = \frac{C(V_1 - V_0) - T(V_1 - V_0)}{C(V_1 - V_0)}$$

where C is the control group; T is the treated group; V_1 the volume before treatment (mm^3); and V_0 the volume after treatment (mm^3).

Transmission electron microscopy (TEM)

The tumor samples were cut into 1 mm^3 blocks and fixed in 4% glutaraldehyde and immersed in Epon 821, and embedded for 72 h at 60℃. The cells were cut into ultrathin section (60 nm) and stained with uranyl acetate and lead citrate. Cell morphology was observed by TEM. made in Japan.

TUNEL assay

The tumor samples were cryopreserved in liquid nitrogen and cut into 8-μm-thick slices. Slices were fixed in ice-cold 80% ethanol for up to 24 h, treated with proteinase K and 0.3% H_2O_2, and labeled with fluorescein dUTP in a humid box for 1 h at 37℃. Slices were then combined with POD-horseradish peroxidase, stained with DAB, and counterstained with methyl green. Controls were treated the same except for labeling with fluorescein dUTP. Cells were examined by light microscopy. The apoptotic index (AI) was calculated as follows: AI = (number of apoptotic cells/total number of cells) × 100%.

Immunohistochemical staining

The tumor samples were cryopreserved in liquid nitrogen and cut into 8-μm-thick slices and fixed in acetone. After washing in PBS, slices were incubated in 0.3% H_2O_2 solution at room temperature for 5 min. Slices were then incubated with anti-Bcl-2 or anti-Bax monoclonal antibody at a 1:300 dilution at 4℃ overnight. After washing in PBS, the second antibody, biotinylated anti-rat IgG, was added and the cells were incubated at room temperature for 1 h. After washing in PBS, ABC compound was added and then incubated at room temperature for 10 min. DAB was used as the chromogen. After 10 min, the brown color signifying the presence of antigen bound to antibodies was detected by light microscopy. Controls were prepared in the same manner as the experimental group, except for incubation with the primary antibody. The positive rate (PR) was calculated as follows: PR = (number of positive cells/total number of cells) × 100%.

Table 1 Inhibition effect of genistein on implanted tumors in nude mice (mean ± SD)

Groups dosage	Number of animals		Volume of tumors (mm^3)		Inhibition rate (%)
	Beginning	Ending	Beginning	Ending	
Control group					
0.2 mL saline	5	5	20.6 ± 1.1	499.8 ± 11.8	
DMSO					
1.5 mg/kg	5	5	20.6 ± 1.3	509.4 ± 8.6	
Genistein					
1.5 mg/kg	5	5	20.3 ± 1.6	458.2 ± 6.7	10.8[a]
1.0 mg/kg	5	5	21.6 ± 1.6	359.7 ± 6.4	29.9[a]
1.5 mg/kg	5	5	21.5 ± 1.6	319.5 ± 10.6	39.6[a]

[a]$P < 0.05$ *vs* the control group.

RT-PCR

The tumor samples were cryopreserved in liquid nitrogen and total RNA was extracted. The concentration of RNA was determined by absorption at 260 nm. The primers for Bcl-2, bax and β-actin were as follows: β-actin (500 bp) 5'-GTGGGGCGCCCCAGGCACCA-3', 5'-CTCCTTAATGTCACGCACGATTTC-3'; Bcl-2 (716 bp) 5'-GGAAATATGGCGCACGCT-3', 5'-TCACTTGTGGCCCAGAT-3'; bax (508 bp) 5'-CCAGCTCTGAGCAGATCAT-3', 5'-TATCAGCCCATCTTCTTCC-3'. PCRs were performed in a 50 μL reaction volume. RT-PCR reaction was run as follows: 94℃ for 7 min, one cycle; 94℃ for 1 min, 72℃ for 1 min, 30 cycles; 72℃ for 7 min, one cycle. Ten microliters PCR product was placed on to 15 g/L agarose gel and observed by ethidium bromide staining using a Gel-Pro analyzer.

Statistical analysis

Datas were analyzed by analysis of variance, and statistical significance was considered at $P < 0.05$.

RESULTS

Inhibitory rate of tumor growth

An inhibitory effect was observed in all therapeutic groups, and the IR for 0.5, 1 and 1.5 mg/kg genistein was 10.8%, 29.9% and 39.6%, respectively (Table 1).

Morphological changes

Control cells had a normal structure, but some cells in the therapeutic groups had apoptotic characteristics, including chromatin condensation, appearance of chromatin

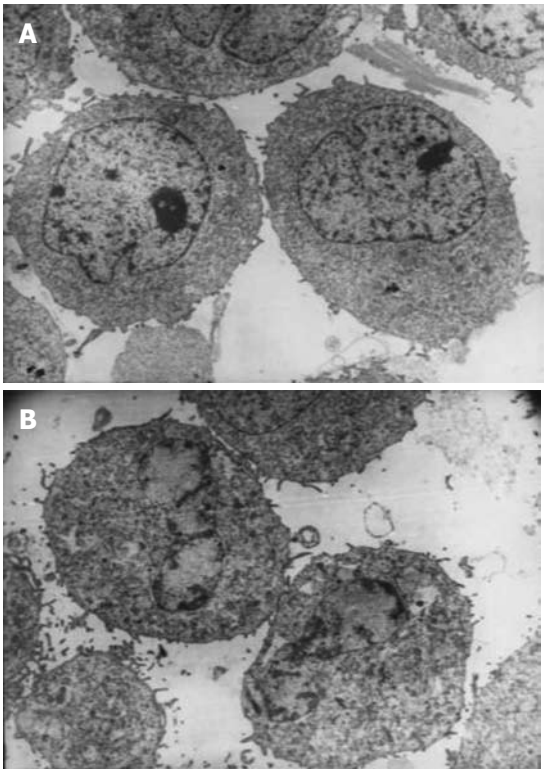


Figure 1 **A:** Ultrastructure of transplanted tumor cells (× 4800); **B:** Ultrastructure of apoptotic transplanted tumor cells induced by genistein (× 4800).

crescents, and nucleus fragmentation, which were seen by TEM (Figure 1A and B) .

TUNEL assay

Positive staining was located in the nucleus. The AI for 0.2 mL normal saline solution, 1.5 mg/kg DMSO, and 0.5, 1 and 1.5 mg/kg genistein was 12.6% ± 0.6%, 13.4% ± 0.7%, 28.9% ± 1.2%, 33.8% ± 1.6% and 37.7% ± 1.2%, respectively (Table 2) .

Expression of Bcl-2 proteins

Positive staining was located in the cytoplasm. The PR for Bcl-2 protein for 0.2 mL normal saline solution, 1.5 mg/kg DMSO, and 0.5, 1 and 1.5 mg/kg genistein was 18.4% ± 1.6%, 17.9% ± 0.7%, 11.9% ± 0.9%, 5.9% ± 0.7 and 4.2% ± 0.6%, respectively by immunohistochemical staining (Table 3) .

Expression of Bax proteins

Positive staining was located in the cytoplasm. PR for bax protein for 0.2 mL normal saline solution, 1.5 mg/kg DMSO, and 0.5, 1 and 1.5 mg/kg genistein was 11.2% ± 0.8%, 11.9% ± 0.5%, 20.9% ± 1.7%, 24.9% ± 0.8% and 29.6% ± 1.7%, respectively (Table 3).

RT-PCR

The density of Bcl-2 mRNA for 0.2 mL normal saline solution, 1.5 mg/kg DMSO, and 0.5, 1 and 1.5 mg/kg genistein decreased progressively, and the density of bax mRNA for 0.2 mL normal saline solution, 1.5 mg/kg DMSO, and 0.5, 1 and 1.5 mg/kg genistein increased progressively, with elongation of time by RT-PCR.

Table 2 Apoptotic index (AI) of implanted tumors in nude mice (%)

	AI
Control group	
0.2 mL saline	12.6 ± 0.6
DMSO	
1.5 mg/kg	13.4 ± 0.7
Genistein	
0.5 mg/kg	28.9 ± 1.2
1.0 mg/kg	33.8 ± 1.6
1.5 mg/kg	37.7 ± 1.2

Table 3 Positive rate of bcl-2 proteins and positive rate of bax proteins of implanted tumors in nude mice (%)

	Positive rate of bcl-2 proteins	Positive rate of bax proteins
Control group		
0.2 mL saline	18.4 ± 1.6	11.2 ± 0.8
DMSO		
1.5 mg/kg	17.9 ± 0.7	11.9 ± 0.5
Genistein		
0.5 mg/kg	11.9 ± 0.9	20.9 ± 1.7
1.0 mg/kg	5.9 ± 0.7	24.9 ± 0.8
1.5 mg/kg	4.2 ± 0.6	29.6 ± 1.7

DISCUSSION

Currently, only a few chemotherapeutic drugs are effective for the treatment of human primary gastric carcinoma, and there is a clear need to look for new anti-gastric carcinoma drugs. Genistein is a planar molecule with an aromatic A-ring, has a second oxygen atom from that in the A ring, and has a molecular mass similar to those of the steroidal estrogens. It has estrogenic properties in receptor binding assays[15,16], cell culture[17,18], and uterine weight assays[19-21]. Genistein inhibits topoisomerase Ⅱ[33], platelet-activating factor- and epidermal growth factor-induced expression of c-fos[34], diacylglycerol synthesis[35], and tyrosine kinases[36]. It also inhibits microsomal lipid peroxidation[22] and angiogenesis[23]. Genistein exhibits antioxidant properties[24-26] and has been reported to induce differentiation of numerous cell types[27-29]. Moreover, a recent report has shown that genistein is a potent cancer chemopreventive agent[30-32].

The Bcl-2 family plays a crucial role in the control of apoptosis. The family includes a number of proteins that have homologous amino acid sequences, including anti-apoptotic members such as Bcl-2 and Bcl-xL, as well as pro-apoptotic members including Bax and Bad[1-4]. Overexpression of Bax has the effect of promoting cell death[10-14]. Conversely, overexpression of anti-apoptotic proteins such as Bcl-2 represses the function of Bax[5-9]. Thus, the ratio of Bcl-2/Bax appears to be a critical determinant of a cell's threshold for undergoing apoptosis[37].

We found that genistein was able to induce apoptosis in SG7901 cells *in vitro*. This apoptosis might have been mediated by down-regulating the expression of the apoptosis-regulated gene Bcl-2 and up-regulating the

expression of apoptosis-regulated gene Bax. In this study, we evaluated the effectiveness of the gastric carcinoma apoptosis induced by genistein *in vivo*, and investigated the molecular mechanisms involved, and the theoretical and methodological basis for the clinical application of genistein, by using an animal model.

We demonstrated that inhibition was induced in all therapeutic groups. Control cells appeared normal in structure, but some cells in the therapeutic groups showed apoptotic characteristics. The AI of 0.5, 1 and 1.5 mg/kg genistein increased with the dose of genistein. Expression of Bcl-2 in the presence of genistein was decreased, but expression of bax was increased. The density of Bcl-2 mRNA decreased progressively with 0.5, 1 and 1.5 mg/kg genistein, and the density of bax mRNA increased progressively. The ratio of Bcl-2/Bax was decreased and triggered apoptosis of transplanted tumor cells. Our results demonstrated that genistein was able to induce apoptosis of transplanted tumor cells in nude mice. The apoptosis may have been mediated by down-regulating expression of apoptosis-regulated gene Bcl-2 and up-regulating expression of apoptosis-regulated gene Bax. Genistein may be potentially used as a chemotherapeutic drug in the anti-gastric carcinoma chemotherapy.

COMMENTS

Background

A recent study has shown that genistein is a potent cancer chemopreventive agent. The anti-tumor activity of genistein might be related to induction of apoptosis of tumor cells, but the precise mechanism of its anti-tumor activity is not well understood. This study investigated the genistein-induced apoptosis of implanted tumors of SG7901 cells in nude mice, the relationship between this apoptosis and expression of Bcl-2 and bax *in vivo*, and the theoretical and methodological basis for the clinical application of genistein.

Research frontiers

Our results demonstrated that genistein was able to induce apoptosis of transplanted tumor cells in nude mice. The apoptosis may have been mediated by down-regulating expression of apoptosis-regulated gene Bcl-2 and up-regulating expression of apoptosis-regulated gene Bax.

Related publications

For more information about each section, please refer to the studies cited in the reference list.

Innovations and breakthroughs

Genistein was able to induce the apoptosis of transplanted tumor cells in nude mice. The apoptosis may have been mediated by down-regulating expression of apoptosis-regulated gene Bcl-2 and up-regulating expression of apoptosis-regulated gene Bax. To the best of our knowledge, no similar studies are available at present.

Applications

According to the results of this study, genistein may potentially be used as a chemotherapeutic drug for gastric carcinoma.

Terminology

Bcl-2: This is a large family composed of various members that are key regulators of apoptosis. High levels and aberrant patterns of Bcl-2 expression have been reported in a wide variety of human caners. Bax: This is a pro-apoptotic member of the Bcl-2 family, which is thought to induce apoptosis. Loss of Bax in genetically engineered mice results in increased tumor incidence, which suggests that Bax may play a role in suppressing tumor growth *in vivo*.

Peer review

The study demonstrated that genistein was able to induce apoptosis of transplanted tumor cells in nude mice. The apoptosis may have been mediated by down-regulating expression of apoptosis -regulated gene Bcl-2 and up-regulating expression of apoptosis-regulated gene Bax. The authors conclude that genistein may potentially be used as a chemotherapeutic drug for gastric carcinoma.

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RAPID COMMUNICATION

Risk factors for intrahepatic cholangiocarcinoma: A case-control study in China

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Abstract

AIM: To carry out a hospital-based case-control study to investigate risk factors for intrahepatic cholangiocarcinoma (ICC) in China.

METHODS: A total of 312 ICC cases and 438 matched controls were included in the study. The presence of diabetes mellitus, hypertension, hepatolithiasis, primary sclerosing cholangitis, liver fluke infection (*Clonorchis sinensis*), was investigated through clinical records. Blood from all participants was tested for hepatitis B surface antigen (HBsAg) and anti-HCV antibodies. Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated using conditional logistic regression.

RESULTS: Compared with controls, ICC patients had a higher prevalence of HBsAg seropositivity (48.4% *vs* 9.6%, $P < 0.000$), and hepatolithiasis (5.4% *vs* 1.1%, $P = 0.001$). By multivariate analysis, the significant risk factors for development of ICC were HBsAg seropositivity (adjusted OR, 8.876, 95% CI, 5.973-13.192), and hepatolithiasis (adjusted OR, 5.765, 95% CI, 1.972-16.851). The prevalence of anti-HCV seropositivity, diabetes mellitus, hypertension, cigarette smoking, and alcohol consumption were not significantly different between cases and controls.

CONCLUSION: These findings suggest that HBV infection and hepatolithiasis are strong risk factors for development of ICC in China.

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Key words: Intrahepatic cholangiocarcinoma; Hepatitis B virus; Hepatitis C virus; Risk factors

INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC), a rare malignant tumor arising from the ductular epithelium of the biliary tree within the liver, is the second most common malignancy after hepatocellular carcinoma (HCC) among the primary liver cancers[1]. It is one of the most biologically virulent malignancies and has a dismal prognosis because of its late diagnosis and absence of effective treatment. There is a marked regional variation in the incidence of ICC, linked strongly to the distribution of risk factors[2]. Therefore, identifying risk factors may lead to prevention and control of this disease. Liver fluke infection (*Clonorchis sinensis* or *Opisthorchi viverrini*)[3,4], primary sclerosing cholangitis (PSC)[5,6], and hepatolithiasis are thought to be the risk factors for ICC[7,8]. Recently, several studies have suggested that HBV and HCV infections, the major risk factors for the development of HCC, also increase the risk of ICC[3,9-13]. However, to the best of our knowledge, no study of ICC and risk factors has yet been conducted in China.

We therefore conducted a hospital-based case-control study to investigate risk factors for ICC in China, a country endemic for HBV infection.

MATERIALS AND METHODS

Subjects

This study consisted of 312 patients with ICC who underwent surgical resection at the Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China, between February 2004 and May 2006. Controls were selected from patients who were unaffected by liver diseases in the Changhai Hospital of the Second

Table 1 Characteristics of cases and controls

Characteristics	ICC (n = 312)	Control (n = 438)	P value
Mean age (SD)	53.2 ± 10.4	54.8 ± 11.8	> 0.05
Gender			> 0.05
Male	207 (66%)	276 (63%)	
Female	105 (34%)	162 (37%)	

Military Medical University, and were matched to cases in terms of age (± 5 years), sex, and date of hospital admission. The control group consisted of 438 patients admitted to the Departments of Gastroenterology, Urology, Ophthalmology, Neurology and Cardiology. All the specimens from ICC were diagnosed by pathological examination.

For both cases and controls, ever having smoked cigarettes was defined as having smoked cigarettes ⩾ 4 d/wk for ⩾ 6 mo, and ever having drunk alcohol as having consumed alcoholic beverages ⩾ 1 d/wk for ⩾ 6 mo.

The presence of diabetes mellitus, hypertention, hepatolithiasis, PSC, liver fluke infection (*C. sinensis*), was investigated through clinical records.

Laboratory tests

Blood samples were taken from all patients on the first morning of hospital admission, and tested for HBsAg and anti-HCV using a commercial ELISA (Abbott Laboratories, North Chicago, IL, USA). The project was approved by the ethics committee at the two hospitals.

Statistical analysis

Categorical variables were compared using the χ^2 test and continuous variables were compared using Student's t test. Odds ratio (OR) and 95% confidence intervals (CI) were calculated using a conditional multiple logistic regression model. The model was constructed using a forward selection process. $P < 0.05$ was considered statistically significant. These analyses were performed using SPSS 11.0 software (SPSS, Chicago, IL, USA).

RESULTS

No differences were found between patients with ICC and controls according to age and gender (Table 1). Table 2 shows the results of univariate analysis on the risk factors for ICC. The prevalence of anti-HCV seropositivity, diabetes mellitus, hypertension, cigarette smoking and alcohol consumption were not significantly different between the patient and control groups. Compared with the controls, ICC patients had a higher prevalence of HBsAg seropositivity (48.4% *vs* 9.6%, $P < 0.000$), and hepatolithiasis (5.4% *vs* 1.1%, $P = 0.001$). None of the patients had PSC. Liver fluke infection was found in 11 ICC cases (3.5%). However, it is difficult to obtain accurate diagnostic evidence for liver fluke infection and PSC in control groups. Thus, we could not compare these two variables between the patients and controls.

All variables in the univariate analysis were further evaluated by multivariate logistic regression analysis. The

Table 2 Risk factors for ICC by multivariate logistic regression analysis

Variables	ICC Cases n=312 (%)	Controls n = 438 (%)	OR (95% CI)
HBV			8.876 (5.973-13.192)
HBsAg+	151 (48.4)	42 (9.6)	
HBsAg–	161 (51.6)	396 (90.4)	
HCV			0.933 (0.281-3.1)
Anti-HCV+	9 (2.9)	6 (1.4)	
Anti-HCV–	303 (97.1)	432 (98.6)	
Diabetes mellitus			1.505 (0.597- 3.796)
Yes	13 (4.2)	11 (2.5)	
No	299 (95.8)	427 (97.5)	
Hypertension			1.492 (0.867-2.570)
Yes	34 (10.9)	38 (8.7)	
No	278 (89.1)	400 (91.3)	
Hepatolithiasis			5.765 (1.972-16.851)
Yes	17 (5.4)	5 (1.1)	
No	295 (94.5)	433 (98.9)	
Cigarette smoking			1.237 (0.7-2.188)
Yes	43 (13.8)	67 (15.3)	
No	269 (86.2)	371 (84.7)	
Alcohol consumption			0.809 (0.487-1.345)
Yes	39 (12.5)	41 (9.4)	
No	273 (87.5)	397 (90.6)	

significant independent risk factors for development of ICC were HBsAg seropositivity (adjusted OR, 8.876, 95% CI, 5.973-13.192) and hepatolithiasis (adjusted OR, 5.765, 95% CI, 1.972-16.851). Other variables were not significant risk factors by multivariate logistic regression analysis.

The mean age was significantly younger in the HBsAg-positive ICC group (50.8 ± 10.4 years) compared with the HBsAg-negative group (55.5 ± 9.9 years).There were more men among HBsAg-positive ICC patients compared with the HBsAg-negative group [76.8% (116/151) *vs* 56.5% (91/161); $P < 0.001$]. Among ICC patients, cirrhosis was found in 69 who were HBsAg-positive (45.7%), and in six who were HBsAg-negative (3.7%).

DISCUSSION

Recently, infection with HCV or HBV has been suggested as a risk factor for ICC[3,9-13]. A case-control (41 cases and 406 controls) study from Korea reported that the prevalence of anti-HCV and HBsAg positivity was 12.5% and 13.8%, respectively, in patients compared with 3.5% and 2.3% in controls. Multivariate analysis found anti-HCV positivity to be associated with ICC (OR, 3.9) but not with HBsAg positivity (OR, 1.3)[3]. A prospective cohort study from Japan showed that 2.3% of 600 patients with HCV-related cirrhosis developed ICC during a mean follow-up of 7.2 years. The risk of developing ICC among patients with HCV-related cirrhosis was 1000 times higher than that in the general population[9]. A case-control study from Italy that compared 26 patients with ICC with 824 controls found that seropositivity for anti-HCV and HBsAg was 25 and 13% in ICC cases and 5.8 and 6.7% in controls, respectively. A statistically significant increase in the OR was observed for anti-HCV (OR, 9.7), whereas a weaker, non-significant association was found with HBsAg (OR, 2.7)[10]. Similarly, according to a case-control study

that involved 625 cases and 90 834 controls from the United States, the prevalence of anti-HCV and HBsAg was 5.6 and 0.2% in ICC cases and 1 and 0.2% in controls, respectively. The adjusted OR for ICC was 6.1 for anti-HCV, whereas no association was found with HBsAg (OR, 0.8)[11]. Another hospital-based case-control study from Japan showed that HCV seropositivity was detected in 36% of ICC patients and 3% of controls, and the adjusted OR for ICC development was 6.02 for anti-HCV[12]. Overall, these studies suggest that HCV infection may play a role in ICC development, whereas HBV infection does not seem to be a relevant risk factor for the disease. Only sporadic cases of ICC have been reported with HBV infection[13]. In the current study, seropositivity for HBsAg and anti-HCV were found in 48.4 and 2.9% of ICC cases, and in 9.6 and 1.4% of controls, respectively. This indicates that HBV, but not HCV infection is a strong risk factor for development of ICC. These findings differed markedly from the results of previous studies[3,9-12]. ICC is characterized by wide variability in incidence and risk factors. HCV seems to be associated with ICC in regions with relatively low prevalence of HBV infection, such as Japan and the United States[9,11]. China, a highly endemic area for HBV infection, has an HBsAg-positive rate of 9.09% in the general population[14], but a low incidence of HCV infection (0.42%-2.1%)[15,16]. Therefore, the difference in etiological factors associated with ICC can be explained by the type of endemic hepatitis virus. Thus, screening and vaccination for hepatitis B may be a useful strategy to decrease the high rate of ICC in China. It is noteworthy that the prevalence of HBV or HCV infection in our controls is compatible with the figure observed in the general population in China, which suggests no relevant selection bias in our controls.

The precise mechanism of HBV infection in the carcinogenesis of ICC is still unknown. Integration of the HBV sequence into the human genome is one of the most important steps in HBV-related carcinogenesis[17]. HBV sequences are found in human[18] and duck[19] bile duct epithelial cells by *in situ* hybridization. HBV sequence integration is suggested to have a direct oncogenic effect by modulating the expression of neighboring cellular genes involved in the regulation of cell proliferation and apoptosis. HBV X gene-encoded protein (HBX) acts as a transactivator of various cellular genes and plays a crucial role in the pathogenesis of HCC[17,20]. Wang *et al*[21] has observed frequent, strong HBX immunochemical staining in bile duct cells of cancerous and surrounding hepatic tissues, in some HBV-infected ICC specimens, and has suggested that HBX may contribute to the pathogenesis of ICC. Furthermore, it has been shown that HBX may activate the transcriptional expression of human telomerase reverse transcriptase, which leads to tumorigenesis in cholangiocytes[22]. Further studies are required to investigate the cellular transformation mechanisms of ICC induced by HBV infection.

Cirrhosis, such as alcoholic cirrhosis and HCV-related cirrhosis, has also been associated with ICC[9,10]. HBV infection may result in chronic hepatitis, followed by liver cirrhosis. Our data showed that cirrhosis was found in 69 HBsAg-positive ICC cases (45.7%). This finding suggests that HBV-related cirrhosis may be an essential stage in the development of ICC, as it is for HCC in China.

Hepatolithiasis was another independent risk factor for ICC development in our study. This result was compatible with several previous studies[7,8,23,24]. The carcinogenesis associated with hepatolithiasis may be a multi-step process in which there are changes from hyperplasia to dysplasia, and finally to adenocarcinoma[25]. Recent studies have demonstrated that several cancer-related genes, including those of *p16*, cyclooxygenase-2, prostaglandin E2, c-met, and caudal-related homeobox gene 2, play an important role in the development and progression of ICC arising in hepatolithiasis[26-30].

In conclusion, this study showed that HBV, but not HCV infection and hepatolithiasis, are strong risk factors for development of ICC in China.

COMMENTS

Background

Intrahepatic cholangiocarcinoma (ICC) is one of the most biologically virulent malignancies and has a dismal prognosis because of its late diagnosis and absence of effective treatment. There is a marked regional variation in the incidence of ICC, linked strongly to the distribution of risk factors[2]. Therefore, identifying risk factors may lead to prevention and control of this disease. HBV and HCV infections have been suggested as risk factors for ICC[3,9-13]. However, no study of ICC and risk factors has yet been conducted in the China, a country endemic for HBV infection.

Research frontiers

We carried out a hospital-based case-control study to investigate risk factors for ICC in China. A total of 312 ICC cases and 438 matched controls were included in the study.

Innovations and breakthroughs

We found that HBV, but not HCV infection and hepatolithiasis were strong risk factors for development of ICC in China.

Applications

Screening and vaccination for hepatitis B may be a useful strategy to decrease the high rate of ICC in China.

Peer review

This is a well performed study that shows that HBV, but not HCV infection and hepatolithiasis are strong risk factors for development of ICC in China. The discussion is well balanced.

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CASE REPORT

An uncommon cause of corrosive esophageal injury

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Abstract

We present an unusual case of corrosive esophageal injury following liquid glue ingestion. The endoscopic findings were tissue sloughing and blackened appearance of the esophagogastric junction, due to caustic esophageal injuries following ingestion of glue containing toluene.

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Key words: Ingested foreign bodies; Corrosive esophagitis

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Pace F, Greco S, Pallotta S, Bossi D, Trabucchi E, Bianchi Porro G. An uncommon cause of corrosive esophageal injury. *World J Gastroenterol* 2008; 14(4): 636-637 Available from: http://www.wjgnet.com/1007-9327/14/636.asp DOI: http://dx.doi.org/10.3748/wjg.14.636

INTRODUCTION

Toluene is an aromatic hydrocarbon commonly used as an industrial solvent for the manufacturing of paints, chemicals, pharmaceuticals and rubber. It is commonly found as a glue in the household. It can be swallowed accidentally, can create a burning sensation, and cause gastrointestinal symptoms such as abdominal pain, nausea, vomiting and hematemesis. Their effect on the mouth, pharynx, larynx, esophagus and the rest of gastrointestinal system is unknown. However, no cases have been reported so far in English-language literature.

CASE REPORT

A 37-year-old man was brought to the surgical emergency department of our institution, reporting that he had been threatened by unknown persons 6 h earlier and forced to ingest liquid household glue (containing toluene) mixed with numerous coins and pins. The patient's main complaints were vague: epigastric pain, nausea and vomiting. He was hemodynamically stable, but uncooperative. He did not smoke, nor was he an alcoholic or a drug addict. There were no signs of trauma or burns on the head and neck, except for a slight excoriation on the neck consistent with attempted strangulation, which proved to be self-inflicted. Chest and abdominal physical examinations were normal, except for moderate tenderness in the upper abdomen, without guarding. Laboratory tests were normal. Plain abdominal radiography revealed multiple sharp opacities in the stomach; in addition to these, an image of some pins was detected in a region consistent with the right lower quadrant (Figure 1). We performed upper gastrointestinal (GI) flexible endoscopy (Figure 2). The endoscopic findings were tissue sloughing and blackened appearance of the esophagogastric junction, due to caustic esophageal injuries, following liquid glue ingestion. An attempt was made to remove the foreign body endoscopically, but without success. The patient was scheduled for emergency surgical exploration.

DISCUSSION

Halogenated hydrocarbons, such as the solvent trichloroethane and methylene chloride, are known to produce hepatic and renal toxicity following chronic exposure, as well as central nervous system (CNS) effects with acute exposure[1], but corrosive GI injury has been never reported up to now. Endoscopic investigation furthermore showed metal pins and coins in the stomach. When approaching intestinal foreign body cases, location of the foreign body is of great importance in terms of management. The choice between outpatient treatment and surgical intervention will depend upon the localization and shape of the object. Although sharp objects pass through the gastrointestinal tract without difficulty in 90% of the cases, they may also be removed using fiberoptic gastroscopy without waiting. Early endoscopy is recommended in cases with objects wider than 2 cm and longer than 6 cm, since their



Figure 1 Gastric overt dense opacities on plain abdominal film.

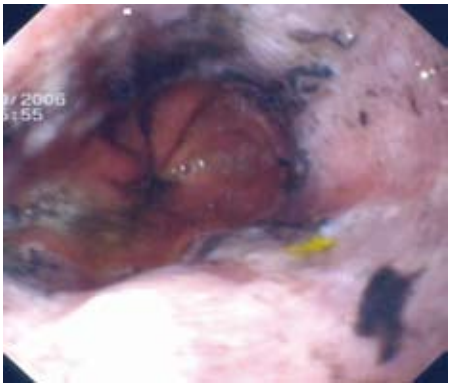


Figure 2 Tissue sloughing and blackened appearance of the oesophagogastric junction.

passage through the pylorus and duodenum might become difficult. Sharp and pointed foreign bodies, as well as elongated foreign bodies, can be very challenging and difficult to manage. It is important to be extremely careful not to make this situation worse or to cause a complication, such as a perforated esophagus, that can be fatal. It should not be considered a defeat to have to remove a foreign body surgically, for this is sometimes the safest means [2]. In this case, the stomach was opened by longitudinal gastrotomy in the greater curvature and the foreign bodies were removed. The next day, on the basis of visual and auditive hallucinations, the patient left the surgical unit, refusing any treatment. A psychotic illness was diagnosed and mandatory treatment was given. The patient was referred for a psychiatric consultation and after one month of follow-up showed a satisfactory recovery.

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CASE REPORT

Ileal schwannoma developing into ileocolic intussusception

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Abstract

Intussusception is rare in adults. We describe a 47-year-old man with ileal schwannoma that led to ileocolic intussusception. Abdominal ultrasonography, abdominal CT scan and barium enema confirmed an ileal tumor. Colonoscopy revealed a peduncular submucosal tumor (SMT) 75 mm long with an ulcerated apex at the ascending colon. The provisional diagnosis was a gastrointestinal stromal tumor of the terminal ileum. Ileocecal resection was carried out and the tumor was histologically diagnosed as schwannoma. Abdominal pain resolved postoperatively. This case reminds us that ileal schwannoma should be included in the differential diagnosis of intussusception caused by an SMT in the intestine.

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Key words: Ileal submucosal tumor; Invagination; Diagnostic imaging; S-100 protein

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INTRODUCTION

Ileal schwannoma is an extremely rare disease, even among small intestine tumors. In Japan, there have been no more than eight cases reported in the literature[1,2]. There have been no reports describing adult cases of ileocolic intussusception due to ileal schwannoma. Here, we describe a rare case of ileal schwannoma that led to ileocolic intussusception.

CASE REPORT

The patient was a 47-year-old Japanese man. He was admitted to our hospital complaining of intermittent abdominal pain of several months' duration that was increasing in both magnitude and frequency. He had not had previous abdominal surgery and had been in good health. No specific family history was identified. His body temperature was 36.4℃, blood pressure was 126/78 mmHg, and radial pulse rate was 66 beats/min and regular. He had neither anemia nor jaundice. A neurological examination revealed no abnormal findings and there was no lymphadenopathy. Routine hematological examination and biochemical tests were within normal limits. Abdominal CT scan revealed a large, comparatively well-defined mass in the colon and intussusception (Figure 1). An air contrast barium enema also showed a large elevated mass 7 cm × 4 cm in diameter with a smooth surface in the ascending colon, which suggested a submucosal tumor (SMT). Colonoscopy revealed a large SMT with an ulcerated apex in the ascending colon (Figure 2). The SMT was peduncular, covered with normal ileal mucosa, and prolapsed from the ileocecal valve to the ascending colon. A biopsy specimen obtained from the lesion showed spindle-like cells. Based on these findings, the patient was diagnosed with intussusception due to intestinal SMT, which suggested a gastrointestinal stromal tumor (GIST). The patient underwent an urgent laparotomy (ileocecal resection) because of the increased frequency of abdominal pain after colonoscopy. The resected tumor, 75 mm × 43 mm × 34 mm in size, was covered with normal ileal mucosa (Figure 3). Histological examination of the resected specimen revealed interlaced bundles of large, bizarre, spindle-like tumor cells without mitotic figures (Figure 4). No fission images were evident. Immunohistological findings were negative for CD34 and α-smooth muscle actin (SMA), and positive for S-100 protein (Figure 4). Based on the above findings, this tumor was diagnosed as ileal schwannoma. The postoperative course was uneventful. No additional therapy was performed. As a result of this treatment, the patient's abdominal pain resolved postoperatively.

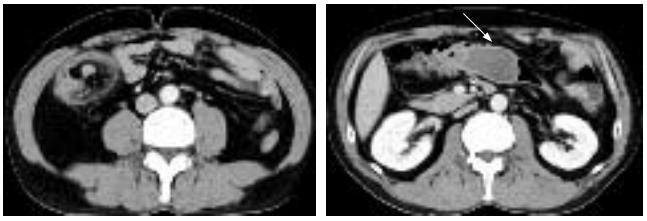


Figure 1 CT revealed a large, comparatively well-defined mass in the colon (arrow), and intussusception.

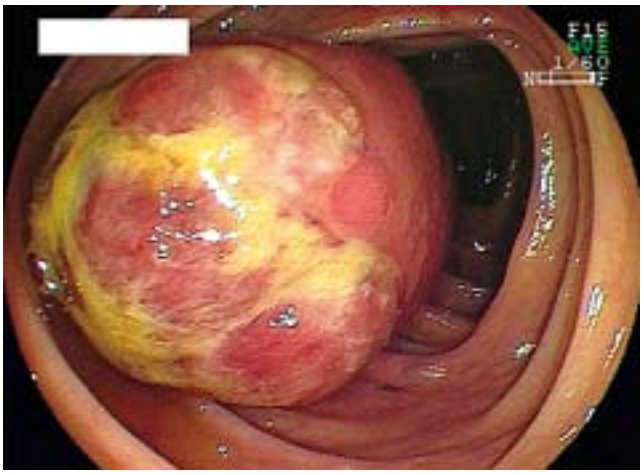


Figure 2 Colonoscopy revealed a large SMT, with an ulcerated apex covered with normal ileal mucosa, in the ascending colon.



Figure 3 Macroscopic findings of the tumor. A 75 mm × 43 mm × 34 mm pedunculated SMT arising from the ileum was observed.

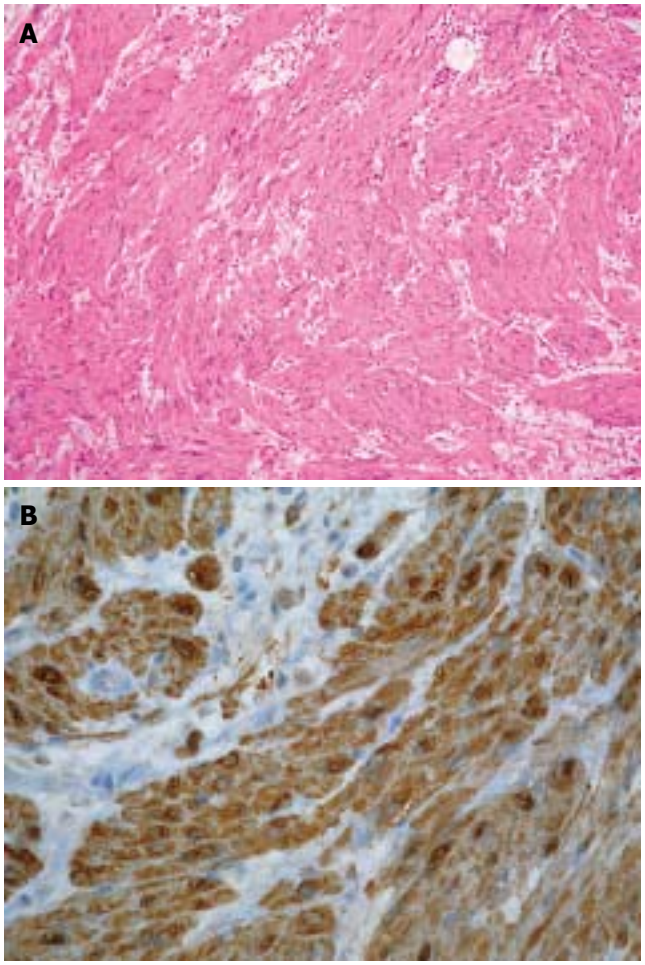


Figure 4 Histological findings of the tumor. **A**: Proliferation of bizarre spindle-like tumor cells without mitotic figures (HE, × 20); **B**: Tumor cells were positive for S-100 protein.

DISCUSSION

Schwannomas are tumors derived from Schwann cells. These tumors are prone to occur peripherally in the head and limbs, and cause radiating pain or sensory paralysis[3]. Although schwannoma rarely occurs on the trunk, even a small one in the brain or spinal cord causes serious neurological symptoms. Manifestations in the gastrointestinal tract are rare, but when they do occur, the incidence is high in the stomach; a schwannoma originating in the small intestine is rare[4]. According to Nozu *et al*[1] and Nagai *et al*[2], a review of the Japanese literature has revealed only eight cases of ileal schwannoma in Japan up to 2003. Common symptoms are abdominal pain and melena[1,2,5,6]; intestinal obstruction or intussusception is rare. In contrast with intussusception in children, nearly all adult cases have primary causes such as a polyp, an SMT or a malignant tumor that needs to be resected[7]. In the present case, the tumor prolapsed to the ascending colon due to intussusception.

The preoperative diagnosis of schwannoma appears to be difficult. According to Nagai *et al*[2], none of the reported cases of ileal schwannoma in Japan had been correctly diagnosed preoperatively. For preoperative diagnosis, CT, ultrasound (US), MRI, and angiography are useful for locating the tumor site. CT depicts the tumor growing outside the wall, and displays a high shadow effect[8]. Imaging of the small intestine shows findings suggestive of an SMT, such as extramural pressure or tumor shadow loss[9]. Angiography of the superior mesenteric artery shows hypervascular tumor vessels[10]. In the present case, CT, US and barium enema images were compatible with schwannoma. However, the diagnosis could not be confirmed despite endoscopy. Because of the increased frequency of abdominal pain after colonoscopy, the patient underwent urgent laparotomy;

thus, angiography and MRI were not performed in the present case.

This disease is treated in principle by surgical resection. It is not possible to differentiate benign from malignant schwannoma confidently with imaging alone. Ileal benign schwannoma is not fatal and patients remain asymptomatic in their daily lives, except for bowel intussusception or obstruction; therefore, it is likely that there are patients with latent small bowel schwannoma, which may be incidentally discovered in the future as a result of advances in diagnostic imaging, such as improved CT and MRI.

In conclusion, we have reported a very rare case of ileal schwannoma that led to ileocolic intussusception. Thus, it is necessary to be aware that an ileal schwannoma may cause ileocolic intussusception and should be suspected in a patient with chronic atypical abdominal pain.

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CASE REPORT

Combined duplication of the colon and vermiform appendix in an adult patient

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Abstract

Combined duplication of the colon and vermiform appendix is one of the rare congenital anomalities of the alimentary tract. Only a few cases have been reported in the adult population. A 28-year-old man presented to the clinic with a mass in the right flank. Imaging showed only a hydronephrotic atrophic kidney. The final diagnosis was only available at exploration. Combined duplication of the tubular colon and vermiform appendix was confirmed histopathologically. The patient was treated with nephrectomy and complete resection of the duplicated colon and vermiform appendix. The patient recovered uneventfully, and has done well for the past year. This is believed to be one of the first reports of combined duplication of the tubular colon and vermiform appendix as a cause of hydronephrotic atrophic kidney in an adult patient.

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Key words: Colon duplication; Appendix duplication; Hydronephrosis; Kidney

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INTRODUCTION

Gastrointestinal duplication is rare, although it may be observed throughout the alimentary tract. Most duplication is located in the ileum and the presenting symptoms are bowel obstruction, intestinal bleeding and/or even perforation. Apart from ileal duplication, colon duplication is a rare congenital anomaly[1-4]. We present a case of combined duplication of the colon and appendix vermiformis, which led to hydronephrotic atrophy of the kidney.

CASE REPORT

A 28-yr-old man with fever (38.9℃) and intermittent right flank pain was referred to the clinic. Anorexia, nausea and vomiting were also noted. Physical examination revealed a palpable mass in the right flank. No sign of peritoneal irritation was noted. He had no history of surgery, urinary tract infection and/or calculi. Leukocytosis and urine infection were only the findings upon blood and urine analysis.

Ultrasonography (US) revealed a heterogeneous and hyperechoic pararenal mass and grade 3 to 4 hydronephrosis. Intravenous pyelography (IVP) was diagnostic for a non-functional right kidney. ^{99m}Tc-diethylenetriaminepentaacetic acid (^{99m}Tc-DTPA) and ^{99m}Tc-dimercaptosuccinic acid (^{99m}Tc-DMSA) nephrograms confirmed decreased right renal function. CT showed hydronephrosis in the right kidney, with a mass adjacent to the right renal pelvis. A continuum of the mass with the colon was noted. Colonoscopy was not diagnostic for intraluminal pathology.

During exploration, a severely scarred and inflamed mass at the inferior medial pole of right kidney was observed, which caused hydronephrosis and atrophy of the right kidney. Nephrectomy was undertaken. The continuum of the mass was followed during dissection. A precast peritoneal window and connection of the mass to the patent cecum was observed. There were two tubular lumens at the base of the cecum. Resection of the mass was completed with careful dissection to spare the normal adjacent structures. Cecal opening at the connection of the mass was closed with simple saturation, and a formal appendectomy was performed. The patient recovered steadily and was discharged from the hospital after 10 d, and he progressed well over the next year.

Gross pathological examination revealed a combined

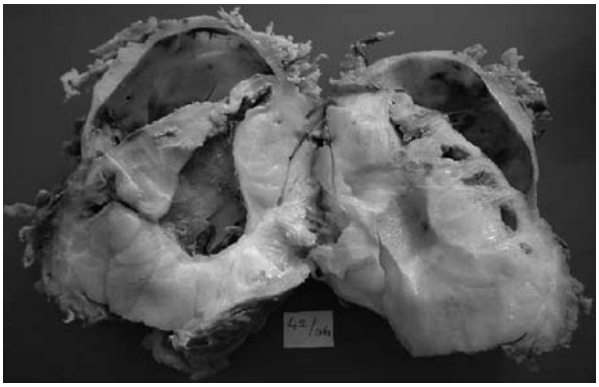


Figure 1 Macroscopic view of the duplicated colon and appendix vermiformis.

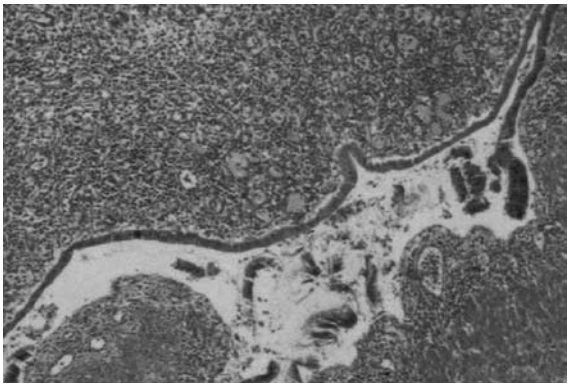


Figure 2 Histological appearance of the duplicated colon and appendix vermiformis.

tubular structure with fecal material inside, and an appendix-like extra tubular structure with an obstructing fecalith in its lumen (Figure 1). This duplicated appendix was not perforated. Histologically, a colonic mucosa layer with accumulation of infiltrating lymphocyte nests, and a duplicated appendix was confirmed. Pathological examination revealed no evidence of malignancy (Figure 2).

DISCUSSION

Gastrointestinal duplication, which may occur throughout the alimentary tract, is a rare congenital anomaly[1]. More than 80% of the cases present before the age of 2 years, as acute abdomen or bowel obstruction[1,2]. Most duplication is located in the ileum and presenting symptoms are bowel obstruction, intestinal bleeding and/or even perforation[3,4]. Apart from ileal duplication, colonic duplication is a rare abnormality. A thorough review of the international literature since 1950 has revealed 83 cases of colonic duplication to date, and only 4%-18% of all gastrointestinal duplications have been reported in the cecum[5-7].

Inclusion of the urogenital tract by an intestinal duplication has been reported in two cases[8,9]. Duplication of the tubular colon and vermiform appendix causing hydronephrotic atrophic kidney in adults has not previously been reported.

Preoperative diagnosis of colon duplication is often difficult. Symptoms usually include abdominal pain, nausea and/or vomiting, palpable mass, weight loss, and bleeding, which often confuses colon duplication with other more common diagnoses. The pathogenesis of these lesions has not yet been well characterized. One of the most widely accepted theories is an abnormality of the embryonic gut that results in the formation of a diverticulum, a cyst, or twinning of a bowel segment[10]. Environmental factors such as trauma or hypoxia during early fetal development have also been suggested to play a role[11]. Although intestinal duplication is considered to be a benign lesion and mostly asymptomatic, they may result in significant morbidity and mortality if left untreated[12]. Indications for surgical intervention often arise in an acute setting, in the form of complications. Specifically, patients with previously undetected duplication may present with bowel obstruction or severe gastrointestinal hemorrhage (i.e., ulcerating gastric mucosa within a duplication cyst)[5]. If encountered incidentally, these lesions should be surgically addressed to avoid any future complication, including the possibility of malignant degeneration within the duplication cyst[5,12].

Duplication of the vermiform appendix is extremely rare. It is found in only 1/25 000 patients (0.004%) operated on for acute appendicitis[13]. The clinical presentation can vary depending on the location of the appendices in the colon. Cave and Wallbridge have classified the observed variants into several types: type A has incomplete duplication with both appendices having a common base; type B has complete duplication with the first appendix arising from its usual location at the confluence of the teniae coli, and the second appendix is located at various sites along the colon; and type C has complete duplication of the cecum, with each part having its own appendix[14,15]. Our case was diagnosed as type B.

Although preoperative diagnosis has been made with the aid of radiological studies such as a barium enema, the majority of cases have been diagnosed at surgery or upon pathological examination. Barium enema studies are considered essential for the diagnosis of tubular colonic duplication, with opacification of two colons being the diagnostic sign. However, in certain cases, barium enema apparently does not show positive findings. A case has been reported in which a barium follow-through study established a complete diagnosis of duplication of the colon and terminal ileum. The findings were confirmed by laparotomy. Although the diagnosis of appendix duplication is a rarity, surgeons should be aware of the possibility, especially when clinical signs and symptoms point to appendicitis, although at laparotomy the appendix looks normal. Routine exploration for a second appendix is definitely not indicated because of its rarity and increased complication rate.

Although duplication of the alimentary tract is rare, the possibility of congenital lesions of the alimentary tract should not be overlooked, even in adults presenting with vague or no gastrointestinal symptoms.

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CASE REPORT

Colonic duplication in an adult who presented with chronic constipation attributed to hypothyroidism

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Abstract

Gastrointestinal duplications are an uncommon congenital abnormality that manifest before the age of two in 80% of cases. Ileal duplication is the most common while colonic duplication, either cystic or tubular, occurs in 10%-15% of cases and remains asymptomatic and undiagnosed in most cases. Mostly occurring in pediatric patients, colonic duplication is encountered in adults in only a few cases. The most common clinical manifestations are abdominal pain and intestinal obstruction. Rarely, duplications present with signs of acute abdomen or acute bleeding. This study reports a case of colonic duplication in an adult who presented with chronic constipation. Complete diagnostic workup was made on several occasions during the previous eight year period, but no pathology was found and chronic constipation was attributed to hypothyroidism caused by long standing Hashimoto thyroiditis. Multislice CT, performed because of abdominal distension, defined colonic pathology but the definite diagnosis of duplication of the transversal colon was made at operation. The cystic duplication and the adjacent part of the ascending and transversal colon were excised en-block. This study implies that colonic duplication, though uncommon, should be included in the differential diagnosis of chronic constipation even when precipitating factors for constipation, such as hypothyroidism are present.

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Key words: Colonic duplication; Constipation; Abdominal distension; Hypothyroidism

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INTRODUCTION

Duplications are rare congenital cystic or tubular abnormalities of the gastrointestinal tract that occur anywhere from the mouth to the anus. Despite a variety of early descriptions, Ladd coined the term alimentary tract duplications in 1937, which described the clinical and pathologic aspects of these lesions more effectively[1]. Their etiology is of embryonic origin and several theories have been proposed. However, none of these theories alone is able to explain the full diversity of these lesions. Although they can present at any age, more than 80% of the cases present before the age of two as an acute abdomen or bowel obstruction[2]. Colorectal duplications occur in 13% of these cases[3]. If encountered incidentally, these lesions should be surgically addressed to avoid any future complications[4,5].

We report a case of colonic duplication in an adult woman who presented with distended abdomen and chronic constipation that was attributed to long standing hypothyroidism.

CASE REPORT

A 42-year-old woman was admitted to the division of gastroenterology with symptoms of significant abdominal distension and chronic constipation. The patient had a medical history of chronic constipation from the early childhood which was not striking until 1991 when, during gestation, significant gaseous bowel distention was found. Complete diagnostic workup was made to find the cause of the constipation but all findings were normal. The constipation was attributed to hypothyroidism caused by long standing Hashimoto thyroiditis treated with thyroid hormone replacement. In 1999 the symptoms progressed and again complete diagnostic workup was made. The diagnosis was elongated colon (dolichocolon). During these eight years the patient used cathartics, different types of tea and manual stool evacuation. There were periods of seven days without stool evacuation. The patient got



Figure 1 Plain X-ray of the abdomen showing a dilated intestinal loop occupying more than half of the abdominal cavity filled with fecal masses.



Figure 2 Abdominal CT showing a dilated intestine from the diaphargm to the pelvis, measuring 36 cm in length with a diameter of 11 cm dislocating normal intestine laterally.



Figure 3 Operative view of normal transversal colon and the distended tubular colonic duplication.



Figure 4 Surgical specimen showing part of the normal ascending and transversal colon together with a tubular duplication with all layers of the colonic wall dividing these structures.

used to irregular bowel habits and after 1999 did not go to regular controls. Finally in the last few months in 2006 she noticed significant abdominal distension and consulted the gastroenterologist. Complete laboratory findings, including proteins, albumins, IgA, IgM, IgG, T3, T4 and TSH were normal. ECG and esophagogastroduodenoscopy were normal. Plain X-rays of the abdomen showed an extremely dilated intestinal loop, presumably colon with fecal masses without signs of obstruction (Figure 1). Colonoscopy revealed normal mucosa with few biopsies showing chronic nonspecific colitis. Anal manometry showed: lower mean resting pressure (50 mmHg), normal squeeze pressure (200 mmHg), normal rectal sensation, rectal compliance and recto-anal inhibitory reflex. The conclusion was that there were no elements for Hirschsprung's disease and that the internal anal sphincter showed slight hypofunction. Multislice CT with peroral contrast showed an enormously dilated intestinal loop from diaphragm to pelvis, measuring 36 cm in length with the diameter of 11 cm occupying the complete abdominal cavity in the midline (Figure 2). The stomach, rectum and genitourinary tract were normal. There were no enlarged lymph nodes. The patient was transferred to the department of surgery and the exploratory laparotomy revealed colonic duplication of the transversal colon with no other intra-abdominal pathology (Figure 3). Duplication was separated by a sharp dissection from the left half of the transversal colon but shared common wall with the right half of the transversal colon. The duplication ended blindly at the splenic flexure and had an opening that communicated with the normal colon at the hepatic flexure (Figure 4). Finally, right hemicolectomy was performed. The patient functioned well postoperatively and a few months after the procedure did not have any stool evacuation difficulties as encountered preoperatively.

DISCUSSION

Gastrointestinal duplications are rare congenital anomalies which in 80% of cases are detected prenatally or in the first two years of life, although they can remain undetected until older age[6]. The clinical picture varies according to their location, size, and other factors such as the presence of ectopic mucosa within the duplication, communication with adjacent bowel, or inflammation. The duplications are classified according to their location (ileum 30%, ileocecal valve 30%, duodenum 10%, stomach 8%, jejunum 8%, colon 7%, rectum 5%), shape: cystic ($\geqslant$ 80%) or tubular[7]. The most common clinical manifestations are abdominal pain and intestinal obstruction. Rarely, such duplications present with signs of acute abdomen or acute bleeding[8]. Pyloric and duodenal duplications can mimic hypertrophic pyloric stenosis or a choledochal cyst[5,9,10]. An ovarian cyst should be considered in female patients, and adolescent patients can sometimes have a presumptive diagnosis of Crohn's disease. Ultrasound is the imaging modality of choice for the evaluation of an abdominal mass in the neonate. Approximately 30% of the intestinal duplications

are diagnosed prenatally[11]. Additional imaging procedures are often required: barium enema, CT scan, radionuclide scan (HIDA scan for choledochal cyst and pertechnetate scan for Meckel's diverticulum). However, gastric, duodenal, and rectal duplications can lead to a diagnostic dilemma.

Chronic constipation as a presenting symptom of intestinal duplication in adults present specific diagnostic problems because of a wide variety of etiologic factors. The cause of constipation is usually multifactorial. Secondary constipation could be caused by low fibre diet, anal fissures, thrombosed hemorrhoids, strictures, and tumors, hypercalcemia, hypokalemia, hypothyroidism, diabetes mellitus, pregnancy, stroke, Hirschsprung disease, Parkinson's disease, multiple sclerosis, spinal cord lesion, Chagas disease, familial dysautonomia, scleroderma, amyloidosis, mixed connective-tissue disease and use of different drugs. Functional constipation could be caused by repressed defecatory urge, irritable bowel syndrome, idiopathic megacolon or megarectum, idiopathic slow transit constipation, chronic intestinal obstruction, anismus, solitary rectal ulcer, intussusception, descending perineum, rectocele or ineffective straining.

Intestinal duplications often require urgent surgical intervention. Secretion of ectopic gastric mucosa causes ulceration of the intestinal mucosa, which can lead to massive bleeding or bowel perforation[12]. Gastric mucosa was found in 50.8% of cystic duplications and was present in all locations, including the colon and rectum with somewhat lower frequency of 33%[3].

Colorectal duplications represent 13% of all duplications[3]. Tubular duplications of the colon, may appear in a double-barreled or Y-shaped form[13,14]. These congenital anomalies have a double muscular layer and epithelium similar to the rest of the colon[13,15]. The clinical signs include constipation, vomiting, volvulus, perforation, and, most commonly, intestinal obstruction owing to the blind end of the duplication compressing the normal bowel[13,14,16]. If there are no other associated malformations, tubular duplications of the colon are often not discovered until there is surgical treatment for the complications.

Most authors recommend that once the diagnosis is made, an elective surgical procedure should be performed to avoid complications and to perform the procedure in an optimal state of the patient, but few think that only symptomatic duplications should be surgically treated. The recommended surgical procedure is excision of the duplication. Intestinal duplications are located on the mesenteric side adjacent to normal bowel. Some duplications and the adjacent normal bowel share part of the muscular coat and the intestinal straight arteries. Thus, resection of both normal and duplicated bowels with intestinal anastomosis is a usual surgical treatment[17]. Sometimes it is possible to resect them without compromising the adjacent bowel vascularization. Although there have been some malignant changes reported in adults[18], colorectal duplications are in themselves benign lesions. For this reason, surgical excision should not be radical but should involve complete resection of the duplication along with the relevant part of the colon[4]. Although rare, colorectal duplications are one possible cause of chronic constipation and, when discovered, should be treated surgically before complications arise. According to previous studies, surgical resection should extend at least 2 cm from the opening of the duplication; because substantial fibrotic changes near the opening of the duplication have been found[4,13]. Prognosis is good because of localized disease and the benign nature of the disease[4].

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CASE REPORT

Bile tract adenomyoma: A case report

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Abstract

This paper described a rare case of adenomyoma of common bile duct. The case is a 51-year-old man who was hospitalized for yellow color skin and sclera and itching for 2 mo without abdominal pain. Nothing special was found in physical examination except yellowish skin and sclera. The clinical presentation and Contrast-enhanced magnetic resonance imaging (MRI), Magnetic resonance cholangiopancreatography (MRCP), and ultrasonography suspected a tumor of the distal bile duct. The patient was treated successfully by pancreaticoduodenectomy. Histologically, the lesion consisted of adenoid and myofibrous tissue and moderate atypia. The immunophenotype of the epithelial component was cytokeratin 7+/cytokeratin 20-. The patient has been well without any evidence of recurrence for 12 mo since his operation.

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Key words: Common bile duct; Adenomyoma; Diagnosis; Pancreaticoduodenectomy

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INTRODUCTION

The benign biliary tract tumor is rarely found in clinical practice. It is only 0.1% among the total biliary tract operations and 6% of extrahepatic biliary tract tumors reported at home and abroad. Till the year 2000, less than 200 cases of benign biliary tract tumor had been reported in English literatures[1], including polyps, adenomatoid papilloma and adenoma, while adenomyoma was seldem found. One patient with biliary tract adenomyoma was admitted to our hospital and reported below.

CASE REPORT

The patient is a 51-year-old man admitted to our hospital in August 2005, who was presented with yellow color skin and sclera and complained of itching for 2 mo. He suffered from anorexia and occasional nausea and deep tea color urine, yellowish white feces and weight loss of 2.5 kg were found, but without vomiting, chill, fever, abdominal pain and distension. Nothing special was found in physical examination except yellowish skin and sclera.

On admission, blood biochemical examination showed: alanine aminotransferase (ALT) 250 U/L, aspartate aminotransferase (AST) 102 U/L, alkaline phosphatase (ALP) 641 U/L, γ-glutamyltranspeptidase (γ-GT) 854 U/L, total bilirubin 32.9 μmol/L; direct bilirubin 22.8 μmol/L; Glucose 4.29 mmol/L, blood urea nitrogen (BUN) 6.56 mmol/L, creatinine (Cr) 67 μmol/L, carbohydrate antigen19-9 42.9 U/mL; carbohydrate antigen-50 57.86 U/mL; carcinoembryonic antigen (CEA) 6.8 ng/mL and γ-GT isoenzyme(-). α-fetal protein (AFP) 6.21 ng/mL, AFU 563 μmol/L.H, ferritin (Fer) 500 μg/L. Contrast-enhanced MRI revealed dilatation of the bile duct and pancreatic duct, eccentric stricture of the distal bile duct and multiple cysts of liver (Figure 1). Magnetic resonance cholangiopancreatograph (MRCP) showed dilatation of the bile duct and pancreas duct, irregular stricture of the bile duct (Figure 2). Ultrasonography showed distension of intrahepatic bile duct (0.32 cm), increase of gallbladder volume (11.1 cm × 4.4 cm) with smooth wall (0.3 cm thick), distension of common bile duct (1.27 cm) but stricture (0.4 cm) at pancreatic segment and a mass (1.6 cm × 0.27 cm) at the lumen of this segment (Figure 3).

Gross observation revealed the tumor was a cord-like mass with smooth surface, white color in resecting surface, wide and immovable base (Figures 4 and 5). Pathological examination demonstrated that the tumor epithelium was adenomyomatosis with adenoid and myofibrous tissue and moderate atypia (Figure 6). Glandular epithelial component expressed cytokeratin 7 (Figure 7) but did not express cytokeratin 20. The stomach, intestine and pancreas were negative after surgery and lymphnodes aside the bile duct and in the hepatoduodenal ligament presented inflammatory hyperplasia. The digestive tract was reconstructed with Child operation. The patient has been well without any evidence of recurrence for 12 mo since his operation.



Figure 1 Contrast-enhanced MRI showing distension of gallbladder, dilatation of the intra and extrahepatic bile duct and eccentric stricture of the distal bile duct, and a mass (arrow) at the lumen of the duct.

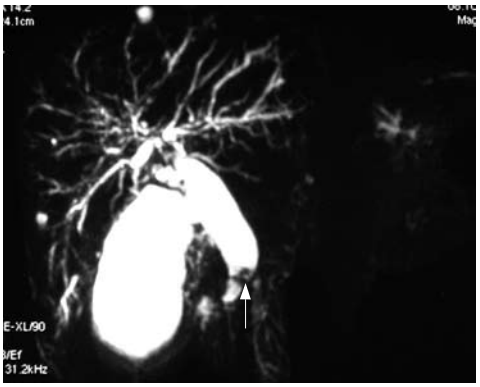


Figure 2 MRCP showing irregular stricture of the distal bile duct and a mass (arrow) at the lumen of the common bile duct.

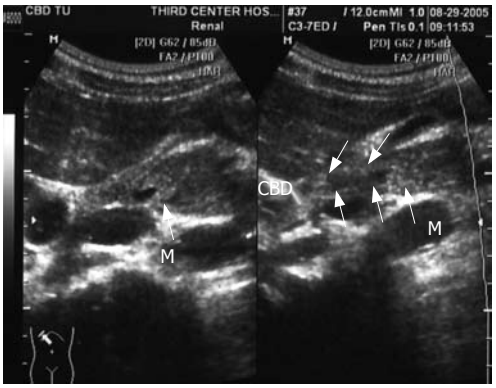


Figure 3 Ultrasonography showing a mass at the lumen of the common bile duct. M: Mass; CBD: Common bile duct.

DISCUSSION

In the year 2000, Beazley and Blumgart[1] reviewed a total of 115 patients with benign polyps, adenomas or cystadenomas. The anatomical distribution of papillomas or adenomas was as follows: most tumors were located in the ampulla or close proximity of the Vaterian system (47%), followed by common bile duct (27%), common hepatic duct (15%), right and left hepatic duct (8%) and cholecyst duct (3%) in frequency. Adenomyoma of the



Figure 4 Gross examination showing a cord-like mass with smooth surface (arrow) and dilatation of proximal part of the common bile duct (a), duodenum (b) and pancreas (c).

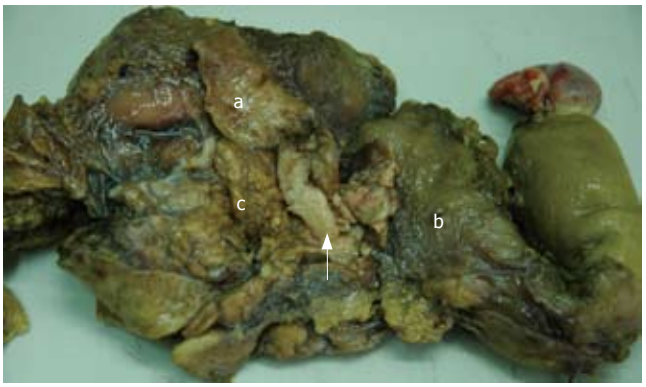


Figure 5 Gross examination of the mass: white color in resecting surface, wide and immovable base (arrow). a: Dilatation of proximal part of the common bile duct; b: Duodenum; c: Pancreas.

biliary tract is a rare nonneoplastic lesion. Abdulkadir Bedirli and his colleagues[2] reviewed the literature from 1964 to 2000, and retrieved a total of 49 articles. Most were case reports of biliary tract adenomyoma. The lesions were located in the gallbladder (n = 35), extrahepatic biliary tree (n = 11), ampulla of Vater (n = 9) and duodenum (n = 4). Histology showed multiple glandular structures surrounded by a fibroblastic/myofibroblastic proliferation[3], and the immunophenotype of the epithelial component was cytokeratin 7+/cytokeratin 20-. The tumor in our case was developed in pancreatic segment of common bile duct, and the immunophenotype of the epithelial cells was cytokeratin 7+/cytokeratin 20-.

It is demonstrated that the adenomyoma is difficult to diagnose preoperatively. The ERCP image can be a useful diagnostic approach as it allows the inspection of the periampullary region and visualization of the bile duct and pancreatic duct. Moreover, a biopsy could be taken from the adenomyoma for further histopathological examination. However, the definite diagnosis is not easy to confirm before operation. Menzel and colleagues[4] showed that the overall accuracy for preoperative histopathological diagnosis was 62% for tumors in ampulla of Vater. The endoscopic retrograde cholangiopancreatography (ERCP) could not be performed in the patient because the mass was invisible in the duodenum[5].

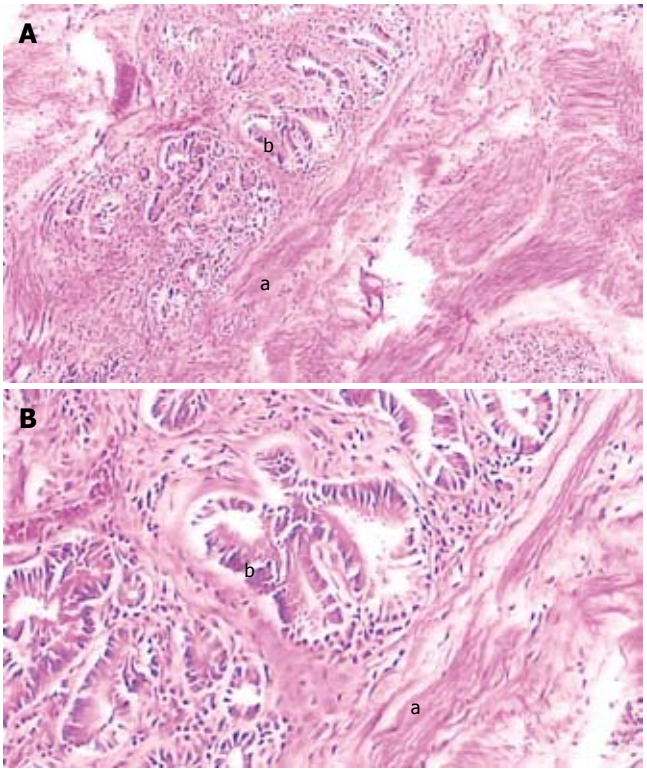


Figure 6 Pathological examination demonstrating the tumor epithelium is adenomyomatosis with adenoid (b) and myofibrous tissues (a) and moderate atypia (b). A: HE, × 100; B: HE, × 400.

Positron emission tomography with [^{18}F] fluoro-2-deoxy-D-glucose was applied to diagnose bile duct cancer[6], and the median standard uptake value (SUV) was 3.8 [confidence interval (CI 3.56-5.01)] for the malignant lesions, 2.9 (CI 2.42-3.22) for the benign lesions, the sensitivity and specificity were 92.3% and 92.9%, respectively.

The adenomas are generally soft, hardly palpable and present little or no resistance in exploring ductal probes, thus being difficult to detect during the operation. They are more frequently detected by intraoperative cholangiography, ultrasonography or choledochoscopy. In the year 2000, Seo[7] reviewed 111 patients with benign or malignant bile duct tumors, analyzed the results of cholangioscopy examinations and classified the findings according to histology of the tumor. Since bile duct tumors exhibit characteristic cholangioscopic findings, cholangioscopy seems to be useful for differential diagnosis.

In a review[8] of 406 cases, brush cytology was used to assess the pancreatico-biliary stricture. There are three adenomas identified on brush cytology but could not be distinguished from adenocarcinoma morphologically.

Intraoperative frozen section from the mass may differentiate whether the lesion is benign or malignant[9,10]. Sato *et al*[11] defined the importance of intraoperative frozen section, Imai *et al*[12] stated that final histological examination should be required to confirm the definite diagnosis.

Therefore, according to the clinical presentation, biochemical, radiographic[13], and endoscopic investigations, and intraoperative frozen section may not be able to confirm whether the tumor is benign or malignant, pancreaticoduodenectomy was sometimes necessary[2,14-16].

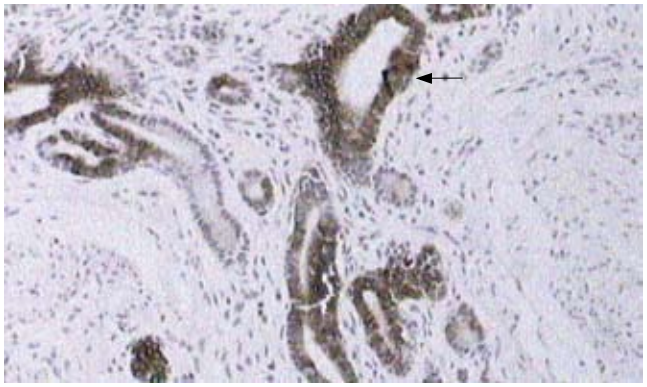


Figure 7 Immunohistochemical features demonstrated that cytokeratin 7 was strongly expressed in the epithelial cells of glandular structures (arrow).

In conclusion, preoperative endoscopic and radiological evaluations, and intraoperative section biopsies are insufficient for differentiating adenomyoma from other malignant tumors. Therefore, extensive surgery has to be performed for some non-neoplastic lesions which are suspected to be malignant.

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Should we redefine large common bile duct stone?

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Abstract

The definition of large stones is not clear ranging from 10 mm to 15 mm and does not include the lower common bile duct (CBD) diameter. Three hundred and four patients who underwent endoscopic retrograde cholangiopancreatography and stone extraction were retrospectively analyzed over a 1-year period. Sixteen patients were different from others in that 10 patients with large stones had stone extraction with a wire basket or a balloon catheter and 6 patients with small stones had stone extraction with mechanical lithotripsy. The definition of large stones should include diameter of the lower CBD and any stone exceeding 2 mm than the lower CBD diameter should be called large stone irrespective of the size of the stone.

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Key words: Large common bile duct stone; Definition

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LETTER TO THE EDITOR

We read with great interest in the article "Small sphincterotomy combined with papillary dilation with large balloon permits retrieval of large stones without mechanical lithotripsy" by Minami *et al* in *World journal of Gastroenterology* published in April 21, 2007[1]. We agree that the size and number of common bile duct (CBD) stones are still a concern in endoscopic sphincterotomy (ES). Balloon dilatation is not suitable for removal of large stones (> 1 cm in diameter) without sphincterotomy[2]. The successful removal of a CBD stone with a maximal diameter with conventional methods depends on the diameter of the distal CBD and the size of the sphincterotomy[3]. In general, common bile duct stones smaller than 1 cm in diameter may be extracted after sphincterotomy using retrieval balloons or baskets, whereas most of the larger stones require lithotripsy prior to their removal. The definition of large stones is not clear ranging from 10 mm to 15 mm[4] and does not include the lower CBD diameter[3].

We retrospectively analyzed patients admitted to our gastroenterology ward in SMS Hospital, Jaipur, between June 2006 and June 2007 who underwent endoscopic retrograde cholangiopancreatography (ERCP) for CBD stones (Table 1). Out of the 304 patients who underwent ES and stone extraction, 16 were different from others in that 10 patients with large stones had stone extraction with a wire basket or a balloon catheter and 6 with small stones had stone extraction with mechanical lithotripsy. The CBD stones were classified into 2 groups according to their size, lower CBD diameter and possible extraction with a wire basket, occlusion balloon or need for mechanical lithotripsy. The patients were divided into two groups: group A with their CBD stones > 10 mm in maximum diameter[2] and dilated CBD, group B with their CBD stones < 10 mm in diameter and their lower CBD diameter being 2-4 mm. We excluded patients with longitudinal stones (longest dimension in the axis of CBD) with their transverse diameter within 3 mm of the lower CBD diameter. All patients underwent ES with their CBD stones extracted using a wire basket or an occlusion balloon. The size of CBD stones was measured by comparing the diameter of the stones with the tip of endoscope, as on the cholangiogram. CBD stones were found in 16 patients with a male: female ratio of 5:11 with a mean age 42.8 years (range 27-58 years). Sixteen patients had abdominal pain, 14 had jaundice, and 5 had cholangitis.

The first group consisted of 10 patients (4 males, 6 females) with a mean age of 44.4 years (range 34-58 years). The median CBD stone diameter was 15.5 mm (range 15-20 mm), the median lower CBD diameter was 16 mm (range 13-24 mm). Eight patients had abdominal pain, 8 had jaundice and 2 had cholangitis. No patient had periampullary diverticula. All patients underwent successful stone removal with a wire basket after undergoing ES. A repeat cholangiography showed no residual stone in the CBD. There was no post procedure complication.

The second group was comprised of 6 patients (2 males, 4 females), with a mean age of 40 years (range 27-48 years).

Table 1 Clinical features, endoscopic retrograde cholangiopancreatography findings and management of common bile duct stone

Number of patients	Age (yr)	Sex	CBD diameter (mm)	Lower CBD diameter (mm)	Abdominal pain	Jaundice	cholangitis	Management
1	37	M	15	16	+	+	-	Stone extraction with basket
2	52	F	20	24	+	-	-	Stone extraction with basket
3	42	F	16	18	+	+	+	Stone extraction with basket
4	58	M	18	15	+	+	-	Stone extraction with basket
5	46	M	15	13	+	+	-	Stone extraction with basket
6	34	F	14	16	+	+	-	Stone extraction with basket
7	40	F	15	18	+	+	-	Stone extraction with basket
8	54	M	17	20	+	-	-	Stone extraction with basket
9	50	F	18	22	+	+	+	Stone extraction with basket
10	33	F	13	14	+	+	-	Stone extraction with basket
11	27	F	8	3	+	+	+	Biliary stent followed by mechanical lithotripsy
12	41	M	8	3	+	+	+	Biliary stent followed by mechanical lithotripsy
13	46	F	8	4	+	+	-	Mechanical lithotripsy
14	42	F	7	3	+	+	+	Biliary stent followed by mechanical lithotripsy
15	35	F	8	3	+	+	-	Mechanical lithotripsy
16	48	F	9	3	+	+	-	Biliary stent followed by mechanical lithotripsy

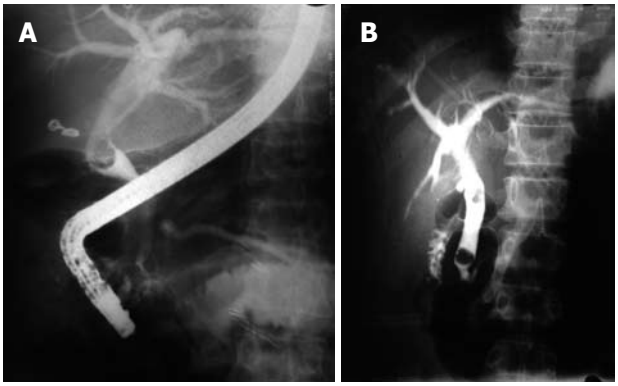


Figure 1 Cholangiogram showing a 8 mm common bile duct (CBD) calculus and a 4 mm lower CBD diameter and intrahepatic biliary radical dilation (**A**), and a 9mm CBD calculus and a 3 mm lower CBD diameter and intrahepatic biliary radicals dilation (**B**).

Six patients had abdominal pain and jaundice, 3 patients had cholangitis. The median CBD stone diameter was 8 mm (range 7-9 mm), the median lower CBD diameter was 3 mm (range 3-4 mm) and all patients had a single stone (Figure 1). No CBD stones were found in any of the patients due to the smaller diameter of the lower CBD. A 7F double pig tail biliary stent was placed followed by mechanical lithotripsy (n = 4) or mechanical lithotripsy (n = 2). One patient had mild post-ERCP pancreatitis which was recovered in 3 d.

In the first group, the overall successful bile duct stone retrieval rate was 100% in the dilated lower CBD. Each patient required one session of ERCP since all of our patients had a single CBD stone.

In the second group, we were not able to remove the CBD stones as their size was disproportionate to the lower CBD diameter. The CBD is a pliable structure but dilates gradually. This is why obstruction occurs even after impaction of stones in lower CBD dilatation. Acute dilatation can only occur after balloon dilatation where the force is transmitted from inside to outward rapidly over a considerable length of the duct, while in removal of CBD stones, the force is exerted on CBD from downward. So, expansion is not expected and will result in injury or avulsion of the CBD and excessive force should not be applied to the stone.

Possible benign stricture was ruled out in all the 6 patients in the second group based on the following points. (a) The patients were asymptomatic with no recurrence of CBD stones during a mean follow-up period of 10 mo (range 6-15 mo). (b) The serum alkaline phosphatase was normal in all the six patients. (c) The common bile duct diameter was normal. Jakobs *et al*[5] reported that patients with a bile duct stenosis have a significantly elevated risk for stone recurrence. This might be explained by bile stasis proximal to the lesion, favoring stone formation. In our study, none of the patients had bile duct stenosis.

In conclusion, the definition of large stones should include diameter of the lower CBD and any stone exceeding 2 mm than the lower CBD diameter should be called large stone irrespective of the size of the stone.

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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 γ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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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.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyr*A, *arg* 1, *c myc*, *c fos*, *etc.*
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[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

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EDITORIAL

Occult sporadic insulinoma: Localization and surgical strategy

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Abstract

Insulinomas continue to pose a diagnostic challenge to physicians, surgeons and radiologists alike. Most are intrapancreatic, benign and solitary. Biochemical diagnosis is obtained and imaging techniques to localize lesions continue to evolve. Surgical resection is the treatment of choice. Despite all efforts, an occult insulinoma (occult insulinoma refers to a biochemically proven tumor with indeterminate anatomical site before operation) may still be encountered. New localization preoperative techniques decreases occult cases and the knowledge of the site of the mass before surgery allows to determine whether enucleation of the tumor or pancreatic resection is likely to be required and whether the tumor is amenable to removal *via* a laparoscopic approach. In absence of preoperative localization and intraoperative detection of an insulinoma, blind pancreatic resection is not recommended.

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Key words: Insulinoma; Occult; CT scan; Endoscopic ultrasonography; Surgery; Laparoscopy

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INTRODUCTION

Although rare, insulinomas are the most common functioning islet cell tumor of the pancreas. Recognition of the key neuroglycopenic symptoms should trigger the initial investigation. Biochemical proof of endogenous hyperinsulinemic hypoglycemia establishes the diagnosis. Several options are available for imaging and localizing these tumors. The tumors are usually small, single, benign, well-circumscribed, and evenly distributed throughout the pancreas. This tumor may be a part of the multiple endocrine neoplasia type 1 (MEN-1) syndrome. Surgical treatment is the only curative method. Patients are almost invariably cured lifelong with complete excision of a benign insulinoma[1-10]. Medical therapy can control hypoglycemic symptoms in approximately 50%-60% of patients with insulinoma, but operation remains the only curative treatment. The urgency of surgery is related to the severity of symptoms and the ability to manage them medically. In general, if hypoglycemia can be managed medically, there is less immediate pressure on the surgeon to find and remove the tumor. However, if symptoms are poorly controlled with medical management, the operation must be successful[5].

Most of the time it is easy to diagnose, localize and remove insulinoma, allowing complete alleviation of the dramatic, and sometimes life-threatening, symptoms of neuroglycopenia. Sometimes, however, this may not be so; despite adequate knowledge of its size and location, an insulinoma may be "occult" (occult insulinoma refers to a biochemically proven tumor with indeterminate anatomical site before operation) and difficult to localize[11-14]. What is (are) the technique(s) of choice to localize insulinoma? What surgical approach will be adopted in case of occult insulinoma? To respond to this important issue, we must discuss the different localization studies with operative procedure options.

EPIDEMIOLOGY OF INSULINOMA

Insulinoma are rare and small neuroendocrine tumors with an incidence of approximately one to four per million per year. It is the most common pancreatic islet cell tumor (25% of endocrine pancreatic tumors) and is malignant in only 5%-11% of cases. Virtually all insulinomas are intrapancreatic in location[1,3,5]. Extrapancreatic insulinomas with symptoms of hypoglycaemia are extremely rare (2%) and are most commonly found in the duodenal wall. In its sporadic form (90%) tumors are generally solitary, whereas in its familial form (5%-10%), they are multiple, especially in the setting of the multiple endocrine neoplasia (MEN) type 1 syndrome. Approximately 80%-90% of insulinomas are less than 2 cm in size and the lesions are distributed equally throughout the head, body and tail of

the pancreas. Most insulinomas are benign (90% of cases). Two per cent of patients have diffuse islet cell hyperplasia, microadenomatosis, or adult nesidiblastosis. Although insulinoma may occur throughout life, the mean age at presentation is 45 (range 8-82) years, and there is a female preponderance (female to male ratio 1.4:1). In MEN 1 the mean age at presentation is younger, at 25 years or less[1-5].

CLINICAL PRESENTATION

Almost all patients with an insulinoma present with symptoms of hypoglycemia secondary to excessive and uncontrolled secretion of insulin[11]. In the case of an insulinoma, symptoms are typically evident in the morning after an overnight fast, and are often precipitated by exercise[15]. Patients learned to avoid symptoms by eating frequent small meals and sugary snacks, with resultant weight gain. Symptoms are often misinterpreted, non-specific, episodic, vary among individuals and can differ from time to time in the same individual. So it is important to obtain a plasma glucose level during symptoms because a normal value in this case rules out the possibility of an insulinoma[5]. Hypoglycemic symptoms can be divided into two categories: neuroglycopenic and neurogenic symptoms[16,17]. Neuroglycopenic symptoms are due to central nervous system neuronal glucose deprivation. They include behavioral changes, confusion, lethargy, weakness, transient motor deficits, difficulty awakening, visual changes, fatigue, seizures and loss of consciousness[18-20]. If hypoglycemia is severe and prolonged, death may result. Neurogenic symptoms are are due to autonomic nervous system discharge caused by hypoglycemia. This results in cholinergic symptoms including hunger, sweating and parasthesia, and adrenergic symptoms including anxiety, tremor and palpitations. Erroneous psychiatric or neurological diagnoses are common in such situations and, on average, it takes up to 2 years before the correct diagnosis is made[5].

CLINICAL EVALUATION

In general, venous plasma glucose levels higher than 3.9 mmol/L after an overnight fast are normal; levels between 2.8 mmol/L and 3.9 mmol/L suggest hypoglycemia and lower than 2.8 mmol/L indicates hypoglycemia. Accurate diagnosis relies upon demonstration of symptomatic hypoglycemia with objective biochemical evidence of cell hypersecretion in the absence of factitious use of an insulin secretagogue. Whipple originally proposed the diagnostic triad of symptomatic hypoglcemia induced by fasting, a blood glucose level below 2.48 mmol/L (0.45 g/L), and prompt relief of symptoms following administration of glucose[5]. A corresponding rise in C-peptide is crucial to verify that insulin secretion is endogenous and to exclude factitia, i.e. self administration of insulin or peroral antidiabetic medication, claimed to be more common than insulinoma. Undetectable C-peptide in a hypoglycemic patient with an elevated insulin level would indicate exogenous insulin administration. Proinsulin levels in normal individuals are usually < 20% of total immunoreactive insulin but tend to be higher in patients with insulinoma. Proinsulin/insulin ratio > 50% may indicate malignant insulinoma, but occurs also with 25% of benign insulinomas. Oral sulphonylurea antidiabetics may cause hypoglycaemia with rise in insulin, C-peptide and proinsulin, and should be excluded by plasma measurement. The use of a ratio of insulin to glucose to aid in the diagnosis has been advocated by some authors but have evidence that they may be misleading. In one series, 34% of patients with excised insulinomas had insulin to glucose ratio > 0.30, demonstrating the fallibility of this ratio. The absolute values of glucose and insulin are the keys: when the glucose level drops to 0.45 g/L, any measurable insulin is abnormal[5,21-23]. A supervised 72 h fast forms the basis for the diagnosis of endogenous hyperinsulinemia[24,25]. The critical pathophysiological feature is failure of insulin secretion to fall to very low rates during episodes of hypoglycemia. From published reports, symptoms develop in 35% of patients within 12 h, 75% within 24 h, 92% within 48 h and 99% within 72 h[22]. The presence of hypoglycemic symptoms with fulfillment of the following criteria is diagnostic of insulinoma: blood glucose level 2.5 mmol/L or lower, insulin level 6 units/mL or higher, C-peptide level 0.2 nmol/L or higher, plasma proinsulin level 0.5 nmol/L or higher, plasma beta-hydroxybutyrate 2.7 mmol/L or lower, change in blood glucose of 0.25 g/L or higher at 30 min after 1 mg intravenous glucagon, and a negative sulphonylurea screen in the plasma and/or urine[21].

DIFFERENTIAL DIAGNOSIS

Hypoglycemia due to excessive endogenous insulin secretion can be caused by a primary pancreatic beta cell disorder including an insulinoma, beta cell hyperplasia or nesidioblastosis (presence of diffuse microadenomatosis, in which multiple small non-encapsulated tumors or nodules are distributed throughout the pancreas), a beta cell secretagogue including a sulphonylurea or a beta cell-stimulating antibody, or an antibody to insulin[20,26-29]. The differential diagnosis of hypoglycaemia includes hormonal deficiencies, hepatic insufficiency, exogenous hyperinsulinism, medication, drugs and enzyme defects. Occasionally differentiating insulinoma from these other causes of hypoglycaemia can be quite difficult. Hypoglycemia was reported in the setting of some tumors and of advanced gastrointestinal stromal tumors. Patients with multiple myeloma or systemic lupus erythematosus (SLE) and hypoglycaemia may have anti-insulin antibodies. These antibodies bind and release insulin in an unregulated manner, resulting in hypoglycaemia. These patients can be distinguished from patients with insulinoma by an anti-insulin antibody test[20,30-32].

LOCALIZATION STUDIES

The localization of small functioning islet cell tumors has proved a challenge to endocrinologists, surgeons and radiologists for several decades. No single imaging test has

emerged as being clearly superior. Imaging to localize insulinomas should only take place once the diagnosis has been confirmed biochemically. The role of imaging is not to diagnose insulinomas, but to identify and localize the tumor. Because virtually all insulinomas are found in the pancreas, it should be the focus of attention; no ectopic insulinomas have been encountered throughout the Mayo Clinic experience[3]. A number of techniques are available to localize an insulinoma, including transabdominal ultrasonography, abdominal computed tomography (CT), magnetic resonance imaging (MRI), arteriography, endoscopic ultrasonography (EUS), transhepatic portal venous sampling, selective arterial calcium stimulation with heptic venous sampling, 111 In-labelled octreotide scan with single photon emission tomography, intraoperative ultrasonography and intraoperative palpation.

Conventional preoperative techniques

Conventional imaging studies such as ultrasonography, computed tomography, and magnetic resonance imaging fail to reveal the majority of insulinomas[5,33-36]. The reported sensitivity of conventional CT scan, MRI, and ultrasonography for detection of pancreatic insulinoma ranges from about 30% to 66%, 37% to 71%, and 9.6% to 61% respectively. Combined Conventional CT scan, MRI and ultrasonography identify 17% to 66% of insulinoma[5,36-39]. With these conventional preoperative techniques, occult insulinoma was present in 31% to 52% of cases in some series[12,13]. The incidence of patients with occult insulinomas seen at the national institutes of health, increased from 16% to 52%[13].

Arteriography was considered the 'gold standard' for insulinoma localization. However, reported sensitivities of 29%-64%, combined with improved non invasive imaging modalities, have decreased its use[33-35,40,41].

Intraoperative techniques

Because of the inability of conventional preoperative studies to localize an insulinoma accurately, some have recommended surgical exploration with intraoperative ultrasonography (IOUS), thereby avoiding other costly preoperative imaging techniques. The most effective method for localizing an insulinoma at surgery is intraoperative ultrasonography[42-48]. Combining many of the attributes of preoperative ultrasonography but also enhanced by the ability to image the pancreas directly without the interference of overlying organs or gas, IOUS has been highly useful in localizing these small tumors. However, it is labour intensive, extremely operator dependent, and, without any definite preoperative localization, obviously focuses the immediate pressure to localize the tumor on the shoulders of the radiologist and ultimately the surgeon. Relevant operative anatomy can be examined to determine the optimal resection method with respect to proximity of the tumor to the main pancreatic duct, combined with colour flow Doppler assessment of adjacent major vessels. It can guide the safest approach to a tumor and facilitate dissection from the pancreatic duct, and also reveal tumor bilobation that has to be appreciated at enucleation. Sensitivities of up to 95% are reported in the detection of pancreatic insulinomas especially useful for tumors in the thicker pancreatic head. Islet cell tumors appear as well-defined, hypoechoic lesions on ultrasonography. The insulinoma is seen as a sonolucent and discrete mass. IOUS is now considered a necessary prerequisite for insulinoma operation together with complete pancreatic exploration and palpation at surgery. The IOUS should be done by an experienced investigator, and can identify tumors larger than 2-3 mm.

Most insulinomas are intraoperatively palpable as a firmer nodule with sensitivity of 90%. Tumors deep within the pancreatic head and in the uncinate process are difficult to palpate, and any lesion is difficult to detect in patients with previous pancreatitis. Combination of IOUS and palpation increases the sensitivity of insulinoma detection to nearly 100%, but requires complete surgical exploration. The head of the pancreas is delivered by the Kocher manoeuvre, with the uncinate process carefully dissected towards the porto-mesenteric vein. The pancreatic tail is mobilized to the mesenteric vein, allowing bidigital palpation and IOUS investigation from both dorsal and ventral surfaces[43,45,48].

Intraoperative laparoscopic ultrasonography greatly facilitates tumor localization and is valuable in guiding the site of pancreatic transection or enucleation especially with respect to the pancreatic ducts. It also defines the relationship of the tumor to the mesenteric vessels and confirms the adequacy of resection. Additionally, it readily demonstrates the relevant operative anatomy, defining the relationship of the tumor to the pancreatic and bile duct, and adjacent blood vessels[49-52].

New preoperative techniques

Transabdominal ultrasonography with frequency probes of 7.5-12 MHz allows higher image resolution. The advent of helical CT scanning has improved the detection of insulinomas compared with conventional CT[5,53-56]. Much investigation has gone into determining the optimal phase for detection and the consensus shows that the use of CT in the early phases is the most sensitive. Diagnostic sensitivity was 94% for dual-phase thin-section multidetector CT, 56% for dual-phase multidetector CT without thin sections and 29% for sequential CT. MR imaging is usually reserved for the detection of small and non-organ-deforming pancreatic tumors, including the functional islet-cell tumors. In current practice MRI is a second-line investigation, but potentially could take over from CT in the future as it becomes more widely available and expertise improves. For some authors, magnetic resonance imaging enables localization of small pancreatic insulinomas[57-62]. For others, while tumor detection increases with tumor size, MRI has been shown to be more accurate than CT, especially for the detection of smaller tumors. Multiple lesions are more difficult to detect using either modality. Current studies still emphasize that MRI is more sensitive for tumor detection. Nevertheless, in many institutions worldwide, CT remains the main imaging method for assessment of pancreatic disease, due largely

to its wide availability.

EUS appears to be emerging as one of the best preoperative studies to identify insulinoma. However, it requires considerable specialized expertise, is observer dependant and is not available in all centers. Sensitivities of up to 94% (range 57%-94%) are reported[63-65]. It has a specificity of 95% for identifying intrapancreatic insulinomas. The combination of biphasic thin-section helical CT and EUS resulted in an overall diagnostic sensitivity of 100%[66]. The EUS appearances of insulinoma are characteristic and include homogenous hypoechoic, rounded lesions with distinct margins. The availability of linear and curvilinear array EUS has broadened its applicability, with the ability to perform fine-needle aspiration cytology of suspicious lesions, contrast-enhanced EUS using Levovist and preoperative marking of lesions to facilitate surgical excision[67-72]. EUS can reveal important relation to the pancreatic duct, and if introduced in the horizontal duodenum allows visualization also of the uncinate process; some of the equipment may be used for tumor biopsy. It has appeared as the most efficient method for preoperative localization, although some isoechoic (6%) or pedunculated tumors may fail to be visualized. Other limitations of EUS include poor evaluation of lesions in the distal body or pancreatic tail[73,74]. Detection rates of 83%-100% of head and body lesions are reported compared with 37%-60% for pancreatic tails lesions[5]. Intraduct ultrasonography has been used to identify small islet cell tumors, and shows promise in differentiating benign from malignant causes of localized stenosis of the main pancreatic duct. It has a reported detection rate of over 90% in the diagnosis of 1-3-mm diameter lesions. However, it is not widely available, requires specialist skills to perform and interpret, and will probably remain confined to selected centres[75,76].

Transhepatic portal venous sampling and selective arterial calcium stimulation with hepatic venous sampling have been proposed as the most sensitive preoperative localization techniques by some authors[77-84]. Selective arterial calcium stimulation with hepatic venous sampling is a more sensitive and specific provocative test, and has largely replaced transhepatic portal venous sampling. This technique has a reported sensitivity of over 90% (range 87.5%-100%) in the accurate localization of pancreatic insulinomas. Although selective arterial calcium stimulation with hepatic venous sampling has a high detection rate, it is not used routinely in most centers as it is invasive, technically demanding and expensive. It may be appropriate when an insulinoma is strongly suspected but all non-invasive imaging tests are negative.

Insulinomas have a low density of somatostatin receptor in only 50% of tumors[85]. Consequently, somatostatin receptor scintigraphy has a limited role in the evaluation of primary insulinomas. Evolving techniques include peptide receptor scintigraphy combined with anatomic imaging methods such as CT or positron emission tomography[57,65]. In tumors that express somatostatin receptor, this can be determined along with detailed axial imaging. This technique has a role in tumor localization, and also for follow-up after treatment with peptide receptor radionuclide therapy[86-93].

PREOPERATIVE LOCALIZATION IS NECESSARY?

Avoiding all efforts at preoperative localization has been espoused by some because a relatively high percentage of insulinomas can be identified intraoperatively in the hands of experienced surgeons. However, reports of insulinomas that remain undetected range from 10% to 27% in several series and preoperative localization was considered essential to successful surgical treatment' in 15 of 41 (37%) patients at one major medical centre with a highly experienced endocrine surgeon. There is absolutely no question that positive localization is required prior to reoperative insulinomas. Preoperative localization is very helpful in planning the operation and certainly offers a measure of comfort to both the surgeon and patient prior to entering the operating room. However, preoperative imaging enables more accurate surgery, which may spare the patient an unnecessary total pancreatectomy and its associated morbidities, and facilitates the detection of metastases. Furthermore, it avoids prolonging the duration of surgery and potential intra-operative damage to major structures such as the splenic vein. It is agreed by the majority, however, that knowledge of the site of the tumor before surgery is helpful in that it allows one to determine not only whether enucleation of the neoplasm or pancreatic resection is likely to be required but also whether the tumor is amenable to removal *via* a laparoscopic approach. It should also mean that the operation itself can be performed more rapidly, thereby reducing its reported morbidity and mortality. Many surgeons emphasize the importance of preoperative localization of insulinomas, because 10%-20% of these lesions cannot be identified during surgical exploration. With recent advances in non-invasive imaging giving a detection rate fast approaching that of intra-operative imaging, the present consensus is generally that preoperative localization is worthwhile. The combination of biphasic thin-section helical CT and EUS resulted in an overall diagnostic sensitivity of 100%[66] and constituted the preoperative localization study of choice.

OPERATIVE MANAGEMENT

Surgical resection is the treatment of choice and offers the only chance of cure. Overall cure rates of 75%-98% are reported after surgery, with prognosis dependant on the stage at presentation and whether complete resection was achieved[94-98]. The surgery may use laparoscopic or open techniques, and includes enucleation, and/or resection. With advances in laparoscopic techniques, both laparoscopic enucleation and resection of pancreatic insulinoma have been performed successfully in many centers, and can be associated with markedly reduced hospital stay and increased patient comfort[99-124]. The operation is facilitated by laparoscopic ultrasonography, but may be complicated-especially in the case of pancreatic head tumors-by higher risk of pancreatic effusion (20%-40%). This operation can be chosen for carefully

selected lesions and should be performed by expert laparoscopists. Enucleation is indicated for small, benign tumors at least 2-3 mm from the main pancreatic duct. Intraoperative ultrasonography can be used to measure the distance between the tumor margin and the main pancreatic duct. Macroscopically, lesions appear reddish-brown in colour in contrast to the surrounding yellowish pancreatic parenchyma, and possess a pseudocapsule with a clear plane of dissection between the tumor and the surrounding soft pancreatic parenchyma. Recent guidelines suggest enucleation is enough if the lesion is clearly localized before surgery, near or at the pancreatic surface, and easily defined intraoperatively. Histologic confirmation of complete excision and the benign nature of the insulinoma are essential[66]. Resection is indicated when the tumor abuts the pancreatic duct or major vessels, or where malignancy is suspected with a hard, infiltrating tumor and puckering of the surrounding soft tissue, distal dilatation of the pancreatic duct or lymph node involvement[102]. Resection options include distal pancreatectomy (with or without splenectomy), pylorous-preserving Whipple procedure, or mid-body pancreatectomy, depending on the site of insulinoma. To determine completeness of tumor excision, a perioperative insulin assay is valuable[125-127]. Intraoperative frozen section can provide information regarding the nature of the tissue, but is often non-diagnostic and cannot assess features of malignancy accurately. Completeness of excision is assessed by gross pathological appearance and should be confirmed by formal histological examination[102]. In 80% of patients with MEN 1 who have endogenous hyperinsulinemia, there are multiple pancreatic tumors. In these patients, an 80%-85% subtotal pancreatectomy to the level of the portal vein with enucleation of lesions in the head of the gland is recommended to reduce the risk of exocrine and endocrine insufficiency. After successful surgical excision, overall 5-year survival has been 97% (5.96); the long-term survival is 88% at 10 years with a higher risk of recurrence in patients with MEN 1[5]. After resection, the risk of recurrence is greater in patients with MEN 1 (21% at 20 years) than those without MEN 1 (5% at 10 years and 7% at 20 years).

OCCULT INSULINOMA

Insulinomas continue to pose a diagnostic challenge to physicians, surgeons and radiologists alike. With the new preoperative localization techniques, especially the combination of biphasic thin-section helical CT and EUS resulted in an overall diagnostic sensitivity of 100%, very few cases of occult insulinoma may still be encountered. In the past, different strategies have been used to deal with this problem. Blind distal pancreatectomy, progressive pancreatectomy and subtotal pancreatectomy with intraoperative glucose monitoring have hitherto been recommended. Blind distal and progressive pancreatectomy are no longer indicated if no insulinoma is found, since recent studies indicate that occult tumors are more commonly located within the pancreatic head[13,42,43,100]. As insulinomas are small and the pancreatic head parenchyma is thick, most non-palpable tumors are in the pancreatic head. Intraoperative ultrasonography can play a critical role in the identification of non-palpable lesions. In a series by Norton[43], only 33% of insulinomas in the pancreatic head were palpable. However, intraoperative ultrasonography correctly identified 100% of them. Still, it is difficult to advocate performing a Whipple procedure on the basis of circumstantial evidence that the tumor may be present in the pancreatic head. If the tumor is not identified despite a careful exploration with intraoperative ultrasonography, termination of the operation without blind resection is recommended. In such cases the surgical procedure should be terminated, the abdomen should be closed, the patient referred to a specialist centre[13,42-44], and subjected to further investigation to verify the biochemical diagnosis and exclude factitious insulin administration. A repeat 72 h fast may be necessary to be certain of the diagnosis. A trial of the best current medical therapy to stabilize the patient on a short-term basis allows time for more extensive localization procedures applied before reoperation, often including the SAS intra-arterial calcium injection test[13,100], a technique that is useful in localizing the region of the pancreas containing the tumor. At re-exploration, if still no tumor is identified, the area of the pancreas suggested by the calcium angiogram should be removed. One might call this an enlightened resection rather than a blind resection.

MEDICAL MANAGEMENT

Medical management of insulinoma is generally restricted to high-risk candidates for surgery, those not suitable for resection or in those who have undergone an unsuccessful operation with persistent symptoms. Options include dietary management, diazoxide, calcium channel blockers and somatostatin analogues. Patients are advised to avoid prolonged fasting and to take frequent meals and snacks, including complex carbohydrates. The recommended daily dose of diazoxide is 200-600 mg orally. Good symptom control is achieved in about 50% of patients. However significant side effects of edema, weight gain, hirsutism and nausea are common. The calcium channel blocker, verapamil, has also been used with some success[102]. Octreotide, a synthetic somatostatin analogue results in only temporary and modest symptom relief. It can worsen hypoglycemic symptoms owing to a grater suppressive effect on growth hormone and glucagon secretion than on insulin. A satisfactory response is reported in only 50% of patients[5,128,129].

CONCLUSION

Insulinomas are uncommon tumors. Most are intra-pancreatic, benign and solitary. A biochemical diagnosis obtained during a supervised 72 h fast followed by high quality dual phase thin section multidetector CT of the pancreas and endoscopic ultrasound confirms the diagnosis and localize the insulinoma in the majority of patients and few cases of occult insulioma may persist. Intraoperative palpation and ultrasonography should enable accurate tumor localization if preoperative studies

fail to localize insulinoma. If the tumor still occult, blind pancreatic resection is not recommended. Laparoscopic treatment is increasingly performed, and leads to long term cure if the tumor is benign.

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REVIEW

Opisthorchis viverrini: The carcinogenic human liver fluke

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Abstract

Opisthorchiasis caused by *Opisthorchis viverrini* remains a major public health problem in many parts of Southeast Asia, including Thailand, Lao PDR, Vietnam and Cambodia. The infection is associated with a number of hepatobiliary diseases, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis. Multi-factorial etiology of cholangiocarcinoma, mechanical damage, parasite secretions, and immunopathology may enhance cholangiocarcinogenesis. Moreover, both experimental and epidemiological evidences strongly implicate liver fluke infection as the major risk factor in cholangiocarcinoma, cancer of the bile ducts. The liver fluke infection is induced by eating raw or uncooked fish products that is the tradition and popular in the northeastern and northern region, particularly in rural areas, of Thailand. The health education programs to prevent and control opisthorchiasis are still required in the high-risk areas.

Key words: *Opisthorchis viverrini*; Carcinogenic; Liver fluke; High-risk areas

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INTRODUCTION

History of Opisthorchis viverrini

Liver flukes are platyhelminth parasites of the class trematoda, and *Opisthorchis viverrini* (*O viverrini*) is a member of the family *Opisthorchiidae*. *Opisthorchis spp.* are prevalent human parasites particularly in the Far East and South East Asia. *O viverrini* is highly prevalent in Thailand and Laos while C. *sinensis* is endemic in South China, Japan, Korea and Taiwan, *O felineus* is the prominent fluke in Eastern Europe[1]. *O viverrini* was first described in the post-mortem examination of two prisoners from a jail in Chiengmai, northern Thailand, in 1911 by Leiper[2] who obtained specimens from Kerr. Kerr[3] reported that 39 of 230 (17%) adult male prisoners examined in a prison in Chiengmai were infected with *O felineus*. In 1927, Prommas[4] identified the worms found at an autopsy of a 17-year-old Thai male residing in Roi-et, northeast Thailand, as *O felineus*. Sadun EH[5] commented that the liver fluke infection in Thailand was caused by *O viverrini*, not by *O felineus*[5], and this was confirmed later in 1965 by Wykoff *et al*[6].

Life cycle of O viverrini

Fish-borne trematodes have a complicated life cycle with two intermediate hosts (Figure 1). Starting from a human host, the adult worms deposit fully developed eggs that are passed in the feces, the eggs from adult flukes are passed out with the faeces. The eggs must get into water in order to hatch and be able to infect their first intermediate host, a freshwater snail. After being ingested by a suitable snail, the eggs release miracidia which undergo in the snail several developmental stages: sporocysts, rediae, cercariae. The snail intermediate hosts are *Bithynia goniompharus, B funiculata and B siamensis*[6,7]. Cercariae are released from the snail and then penetrate freshwater fish that are the second intermediate host (*Cyclocheilichthys spp.*, *Puntius spp.*, *Hampala dispa*), encysting as metacercariae in the muscles or under the scales. Cats, dogs, and various fish-eating mammals including humans are the definitive host[8]. They become infected by ingesting undercooked fish containing infective metacercariae. In infected definitive host, the metacercaria excyst in the duodenum and ascend through the ampulla of Vater into the biliary ducts, where they attach and develop to adults, which lay eggs after 3 to 4

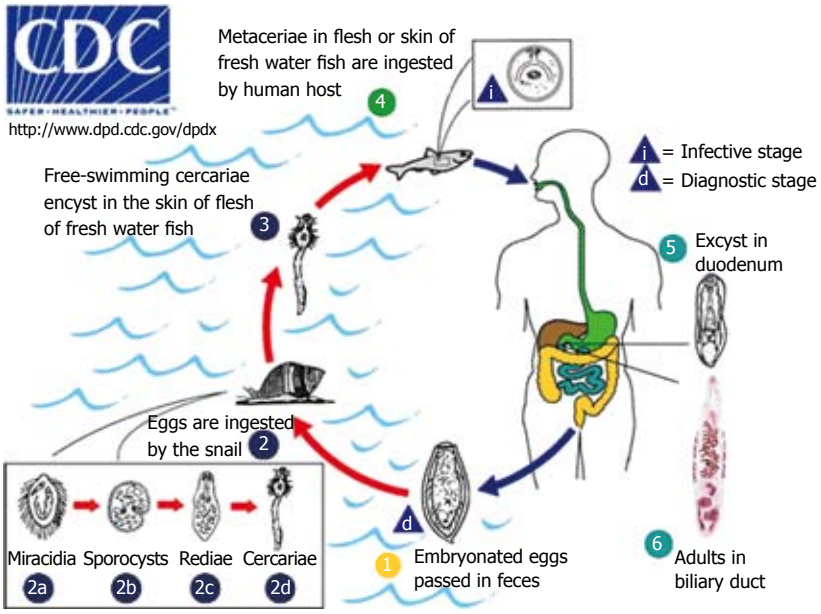


Figure 1 Life cycle of *O viverrini*. Embryonated eggs are discharged in the biliary ducts and in the stool (1). Eggs are ingested by a suitable snail intermediate host (2). Each egg releases a miracidia (2a), sporocysts (2b), rediae (2c), and cercariae (2d). The cercaria is released from the snail and after a short period of free-swimming time in water, it penetrates the flesh of a freshwater fish, where it encysts as a metacercaria (3). Humans are infected through ingestion of undercooked or raw freshwater fishes (4). The metacercaria excysts in the duodenum (5) and ascends the biliary tract through the ampulla of Vater. Maturation to adulthood takes approximately one month (6). (Adapted from: http://www.dpd.cdc.gov/DPDx/HTML/Opisthorchiasis.htm).



Figure 2 Adult worm of human liver flukes: a: *O viverrini*; b: *O felineus*; c: *C sinensis*. The average size of *O viverrini* worm is 7.0 (5.4-10.2) x 1.5 (0.8-1.9) mm[8]. (Adapted from Ash, 1997).

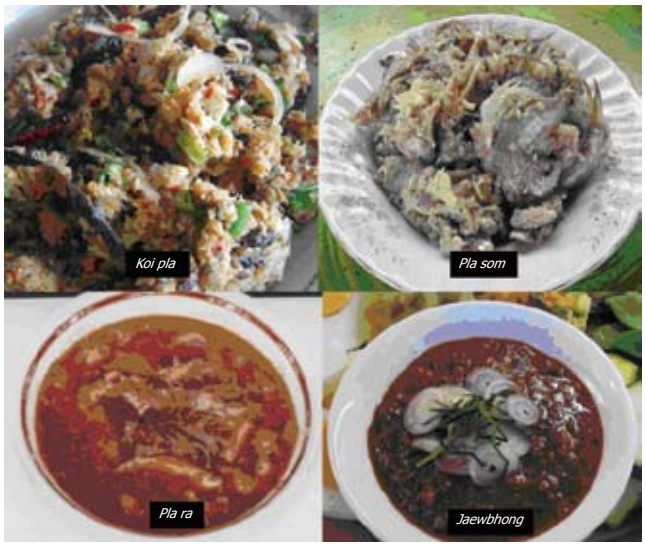


Figure 3 Preparation of *koi pla*, *pla som*, *pla ra* and *jaewbhong*. *koi pla* is prepared with the fish-based meals with local herbs, spices, condiments, rice, and vegetables. *Pla som* is moderately fermented or stored for a few days to weeks. *Pla ra* is fermented fish sauce, popular in Northeastern Thai cuisine. It is made by combining fish, salt, and roasted rice, fermenting in a pot for one to three months. *jaewbhong* is made by combining *pla ra* and chili.

wk. The adult worms reside in the biliary system of the mammalian host, where they attach to the mucosa. The life span of *O viverrini* in human is not known, however, it may be over 25 years as recorded in *C sinensis*.

Morphology of O viverrini

The *O viverrini* are dorso-ventrally flattened, lancet-shaped, thin and transparent. The adult worms are monoecious and the average size is 7.0 (5.4-10.2) × 1.5 (0.8-1.9) mm. The oral sucker is subterminal and the ventral sucker at approximately anterior one-fifth of the body length. Two testes are deeply lobed, diagonal, situated near the posterior extremity (Figure 2)[9]. *O viverrini* is morphologically similar to *C sinensis* but it is slightly smaller in size, the main difference from *C sinensis*. The long, slightly coiled seminal vesicle terminates in the ejaculatory duct, which opens through the genital pore immediately in front of the ventral sucker. Cirrus sac and cirrus are absent. The multilobated ovary is in front of the anterior testes and nearby is the seminal receptacle and Laurer's canal. The vitellaria consist of numerous follicles disposed as several columnous groups in the lateral fields between ventral sucker and testes. Excretory bladder is long, sac-like tubed, S-shaped and runs between the two testes[8].

Epidemiology of O viverrini

In Thailand, at least 15 species of cyprinoides' fish, the freshwater fish, are able to serve as intermediate hosts of *O viverini* metacercariae. The tradition of eating raw or uncooked fish products is the main reason that liver fluke is a problem in Thailand. This is very popular in the northeastern and northern region, particularly in rural areas. In the northeast, three types of preparations contain uncooked, small and medium-sized, fish: (1) *koi pla* which is eaten soon after preparation; (2) moderately fermented

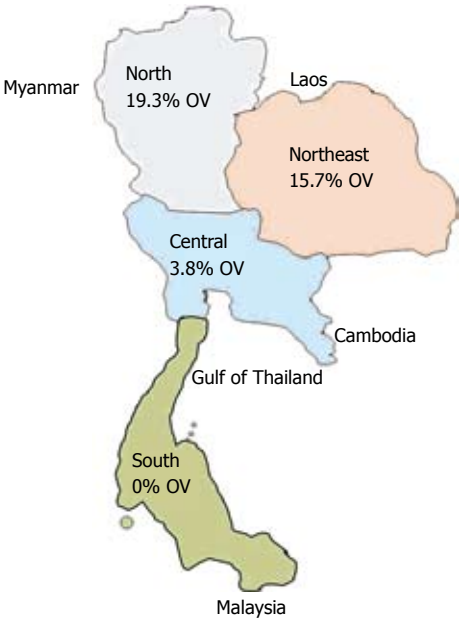


Figure 4 Prevalence of *O viverrini* infection in Thailand[15], OV: Percentage of *O viverrini* infection.

pla som which is stored for a few days to weeks; and (3) *pla ra* which is extensively fermented, highly salted fish, and stored for at least 2-3 mo[5,10] (Figure 3). The consumption frequency of *koi pla* in the population of some communities who ate it every week was approximately 80%[11]. Studies found the highest prevalence of liver fluke infection in the northeasterners who ate *koi pla*[12,13]. The frequencies of *koi pla* consumption have declined and are generally confined to special social occasions, while other under-cooked fish preparations like *pla som* and other moderately preserved fish are generally eaten several times a week[10]. *Pla ra* and *jaewbhong*, fully preserved fishes, are an important staple food and consumed daily by 60%-98% of northeasterners and lowland Laotians[11,14]. Studies have reported that *Koi pla* is probably the most infective, followed by the fish preserved for < 7 d, then *pla ra* and *jaewbhong* in which viable metacercaria are rare[10].

A small scale helminthiasis control program started in 1950 included the opisthorchiasis control in some high-risk areas. Following a number of studies and trial projects of the Faculty of Tropical Medicine, Mahidol University, the National Liver Fluke Control Program has been developed and operated under several National Public Health Development Plans. Presently, the program is being operated in some provinces of the Central, and all provinces of the northeast and north of Thailand[15]. The main liver fluke control strategies comprise of three interrelated approaches, namely stool examinations and treatment of positive cases with praziquantel for eliminating human host reservoir; health education for promotion of cooked fish consumption to prevent infection; and the improvement of hygienic defecation for the interruption of disease transmission[15]. The prevalences of *O viverrini* infection in the north (19.3%), the northeast (15.7%), the central (3.8%) and the south (0%) have recently been reported (Figure 4)[5,15]. The decline in the northeast from 34.6% in 1981 to current level[16,17] is attributed to intensive and continuous control activities[15]. Sithithaworn *et al*[10] performed a cross-sectional survey of intestinal helminthes infections by examining the fecal samples collected from 332 rural northeast Thais from 3 communities during October-November 2000, and they found that the average prevalence of *O viverrini* infection was 14.2% (range 8.6%-19.4%)[10]. Similarly, the prevalence of *O viverrini* infection in Khon Kaen Province, northeast Thailand was found to be 24.5%, which was rather higher in men (27.6%) than in women (21.4%)[18]. A total of 479 stool specimens were collected from rural communities of Ubon Ratchathani Province, Thailand and examined by using two techniques: the modified Kato thick smear and the direct smear. The prevalence of *O viverrini* was 14.8%, being much higher than other intestinal parasite infections[19]. *O viverrini* is still prevalent and a serious health problem in some parts of Thailand. Therefore, the health education and promotion is still more required.

Pathology and pathogenesis of O vivierrini

Most *O viverrini* infection is asymptomatic. Mild symptoms include dyspepsia, abdominal pain, constipation or diarrhea. With chronic infection, the symptoms can be more severe, and hepatomegaly and malnutrition may be present. In rare cases, cholangitis, cholecystitis, and cholangiocarcinoma (CCA) may develop. The severity of the pathology appears to be associated with both intensity and duration of infection. In humans, *O viverrini* inhabits mainly in the intra- and extrahepatic bile ducts and, rarely, in the gall bladder and pancreatic duct. The infection can induce several pathologic changes in the liver, gall bladder and extrahepatic bile ducts. Its infection can enlarge the liver and the weight of the liver may be more than the normal up to 3000-3500 g or more in heavily infected cases. The subcapsular bile ducts of heavily infected patients are usually dilated and show prominent fibrotic wall[20-22]. Microscopically, the intrahepatic lesions of opisthorchiasis are confined to the biliary tree, particularly to the large and medium-sized bile ducts where the flukes are harboured[21]. The typical histologic changes of intrahepatic bile ducts in liver fluke infection include inflammation, epithelial desquamation, goblet cell metaplasia, epithelial and adenomatous hyperplasia and periductal fibrosis[20-23].

Periportal and periductal fibrosis appeared as the most prominent histologic feature in chronic *O viverrini* infection in hamster. This fibrosis correlates with the marked increase in synthesis of hepatic content of type I and, particularly, type Ⅲ collagen as demonstrated in long-term *O viverrini* infected hamsters. Periductal inflammatory cell infiltration of lymphocytes, monocytes, macrophages, eosinophils and plasma cells was also seen both in infected hamsters[24,25] and in human[21]. Granulomatous inflammation in response to the entrap parasite eggs in the periductal tissue and liver parenchyma was occasionally observed in human[26] but frequently seen in animal model[24]. Histologically, the changes seen in the gall bladder and extrahepatic bile duct in opisthorchiasis are adenomatous hyperplasia, epithelial hyperplasia and chronic inflammation[21,27,28]. However, the studies of 189 cholecystectomy specimens in Khon Kaen revealed that

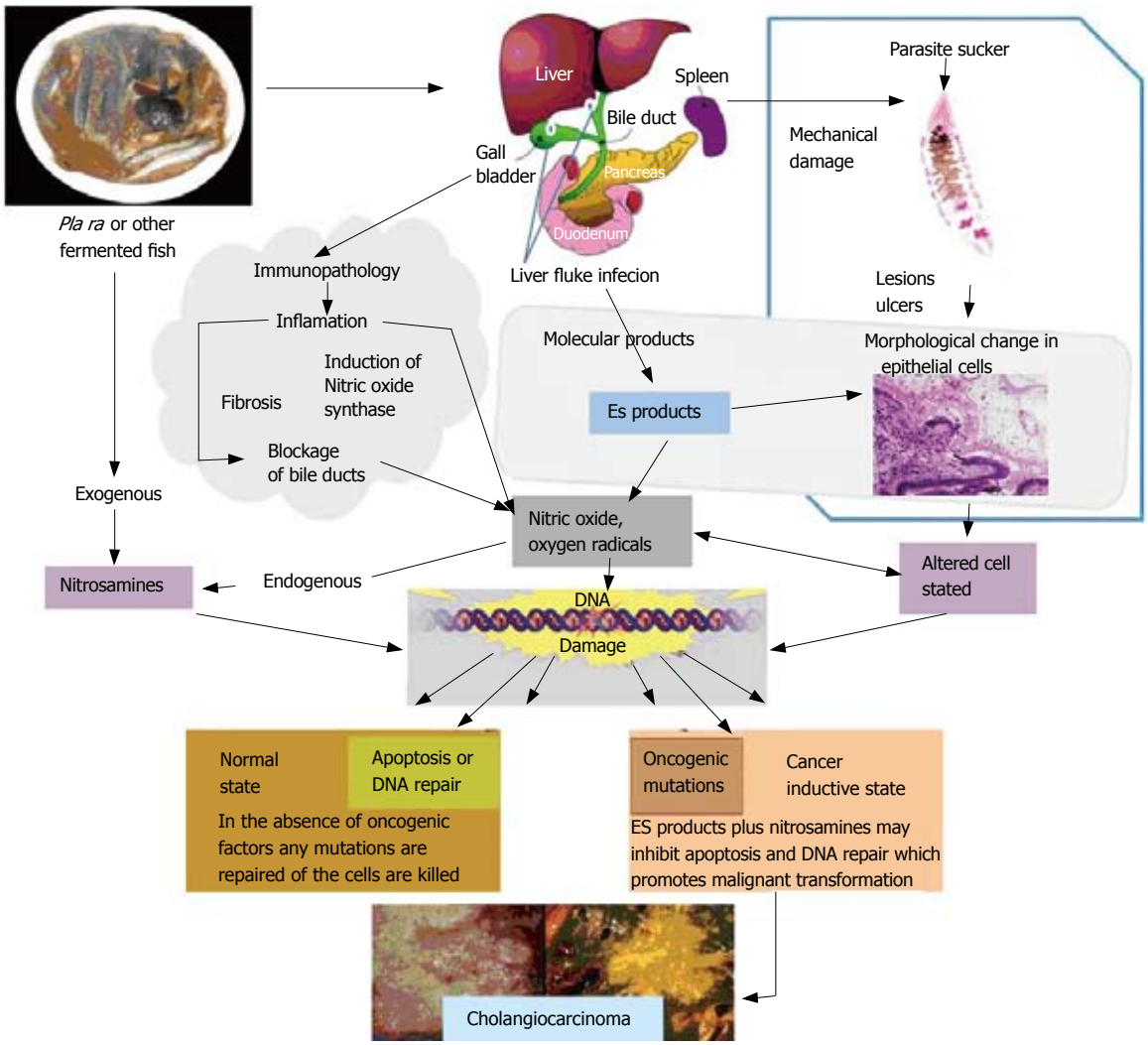


Figure 5 Proposed mechanisms of *Opisthorchis-derived* CCA initiation. (Adapted from Sripa *et al*[30]).

fibrosis of the gall bladder wall the most common finding, among several other histopathologic features, associated with opisthorchiasis as evidenced by parasite-specific antibody. For the extrahepatic bile duct, thickened cystic duct has been noted in autopsies with opisthorchiasis. Hamsters infected with *O viverrini* also showed heavy inflammatory cell infiltration and periductal fibrosis[29].

Multi-factorial etiology of CCA, mechanical damage, parasite secretions, and immunopathology may enhance cholangiocarcinogenesis (Figure 5)[30,31]. The pathogenesis of *O viverrini*-mediated hepatobiliary changes may be due to mechanical irritation caused by the liver fluke suckers and/or its metabolic products[18,24]. However, several authors suggest that immunopathological process may contribute to the hepatobiliary inflamation and damage[22,24,26,27]. Many pathological features in the hamster model reveal evidence of such mechanism, e.g. periportal infiltration of lymphocytes and macrophages. Acute damage may be induced by parasite factors, but the progressive changes are consistent with immunopathologic mechanisms[22]. During liver fluke infection, inflammation, periductal fibrosis, and proliferative responses, including epithelial hyperplasia, goblet cell metaplasia, and adenomatous hyperplasia, may represent predisposing lesions that enhance susceptibility of DNA to carcinogens[32-35]. Several *N*-nitroso compounds and their precursors occur at low levels in fermented food, such as preserved mud fish paste, *pla ra*, a condiment that is a ubiquitous component of the cuisine of northeastern Thailand and Laos[30]. Some authors hypothesised that *N*-nitroso compounds are a primary carcinogen leading to CCA in humans in northeastern region[36,37]. Jinawath *et al*[38] showed the selective up-regulation of genes involved in xenobiotic metabolism in Thai patients with CCA, implying that these genes may be involved in the detoxification of possible carcinogens, such as nitrosamines. In hamster experimentally infected with *O viverrini,* CCA was induced by exposure of sub-carcinogenic doses of nitrosamine[34]. Liver fluke infection can induce the endogenous nitrosation both in humans and experimental animals. Infected hamster showed nitric oxide synthase (NOS) expression by immune effector cells in the inflamed areas and increased endogenous nitrosation of thiazolidine-4-carboxylic acid (thioproline)[39]. In human studies, infected cases showed a higher endogenous

nitrosation than uninfected cases[40,41]. Infected cases had increased levels of plasma and urinary nitrate and nitrite compared to the uninfected cases[39]. Praziquantel treatment and co-administration of ascorbic acid with proline showed enhanced immune responses to *O viverrini* and an increase of endogenous nitrosation[42]. Thus the aforementioned findings clearly demonstrate both exogenous and endogenous nitrosation may lead to DNA alkylation and deamination in predisposed and inflamed tissues[43-45].

Molecular biology of O viverrini

Information regarding the molecular biology of *O viverrini* is limited in comparison of other known trematode parasites. The ribosomal DNA of *O viverrini* has been identified, showing the total length of approximately 13 kb consisting of 4.2 kb of large subunit rDNA, 2.0 kb of small subunit rDNA, 1.0 kb of transcribed spacer DNA and 5.8 kb of non-transcribed + external transcribed spacer DNA. The rDNA units represent 6.1% of the total genomic DNA. The small subunit gene is 1992 nucleotides long and has a G + C content of 50.94%. The use of the rRNA gene sequence comparison for determination of phylogenetic classification of *O viverrini* is comparable to conventional taxonomic classification[46,47]. The mitochondrial cytochrome c oxidase subunit I (CO I) gene and the second internal transcribed spacer region (ITS Ⅱ) gene of *O viverrini* were compared among *O viverrini* from various areas in northeast Thailand. In the nucleotide sequences of partial CO I gene (417 bp), the intraspecific variations were classified into 5 patterns but no area-specific pattern was observed. Nucleotide sequences of a region of the *O viverrini* ITS Ⅱ gene (296 bp) from different areas were different from those of *C sinensis*, *Haplorchis taichui, H pumilio, F gigantica, Echinostoma malayanum and Centrocestus sp*[48]. DNA methylation and demethylation processes are thought be taken place in *O viverrini*. The *O viverrini* genome contains a highly repeated DNA element which is very specific to the organism[49]. The construction of DNA probe to detect genomic DNA of the parasite, for diagnosis of suspected patients or identification of parasites in suspected specimens from endemic areas was developed. The construction of specific DNA probe *O viverrini* was achieved using a unit of highly repeated DNA element (334 base pairs)[50]. The specific DNA probe was characterized and used for detecting the presence of eggs DNA in stool samples[51,52]. Specific primers were designed and used for PCR-based detection. The PCR detection was highly specific and 100% positive. Further studies on molecular biology of *O viverrini* are needed to better understanding of the parasite and host-trematode relationship. Several other molecular studies of *O viverrini* have been reported, including cloning and expression of glutathione S-transferase genes[53,54].

Laha *et al*[55] reported the gene discovery effort for *O viverrini* that should expedite molecular studies of cholangiocarcinogenesis and accelerate research focused on developing new interventions, drugs and vaccines, to control *O viverrini* and related flukes. Approximately 5000 randomly selected cDNAs from the adult stage of *O viverrini* were characterized and accounted for 1932 contigs, representing about 14% of the entire transcriptome, and, presently, the largest sequence dataset for any species of liver fluke. Twenty percent of contigs were assigned GO classifications. Abundantly represented protein families included those involved in physiological functions that are essential to parasitism, such as anaerobic respiration, reproduction, detoxification, surface maintenance and feeding. A total of 164 *O viverrini* contigs contained ORFs with signal sequences, many of which were platyhelminth-specific, such as convergent evolution between host and parasite secreted/membrane proteins identified were homologues of vaccine antigens from other helminths. Finally, ORFs representing secreted proteins with known roles in tumorigenesis were identified, and these might play roles in the pathogenesis of *O viverrini*-induced CCA[55]. One of candidate gene, cysteine protease was studied by Kaewpitoon *et al* (unpublished data). By using specific fluorogenic substrates, majority of *O viverrini* protease was cysteine protease cathepsin L whereas serine and aspartic protease showed very low activities. Molecular cloning of cysteine protease encoding gene by RACE-PCR using degenerated oligonucleotide primers derived from conserved active site obtained a full-length mRNA of 981 bp encoding for 326 amino acids (GenBank, accession number AY821800). The cDNA of the novel *O viverrini* cysteine protease was designated *Ov*-cp-1. Sequence analysis showed high homologies with cysteine proteases of other trematodes, such as *Clonorchis sinensis* (85%) and *Paragonimus westermani* (55%). *Ov*-cp-1 cDNA expression was observed in various developmental stages of *O viverrini* including the metacercariae, juvenile (1, 2, 3 wk), adult worms, and eggs. Polyclonal antibody to *Ov*-CP-1, expressed protein, showed strong reaction with the gut epithelium, testes, vitelline glands and eggs in the uterus of the adult worm. Further functional study revealed that *Ov*-CP-1 could digest human hemoglobin and the activity was inhibited by the addition of E-64. The results suggest that the recombinant *Opisthorchis* cysteine protease *Ov*-CP-1 is potential for development of host-parasite relationship.

Immunity to O viverrini infection

O viverrini infection can induce both cellular and humoral host immune responses. Humoral immune response has been reported in both humans and animal model. The parasite antigens were detected in the epithelial lining of intrahepatic and extrahepatic bile ducts as early as d 3 after hamsters were infected with *O viverrini* and stimulated the host immune system[25]. The antibody levels in the patients with severe opisthorchiasis were significantly higher than those with mild infection. The infected hamster serum contained a component, possibly specific immunoglobulin(s) capable of reacting with adult, juvenile, excretory-secretory (Es) and egg antigens[56-61]. Antibody response to both somatic and Es antigens were detected[60,62]. The antibody responses in hamsters were first detected as early as 7-14 d post-infection. During early infection, hamsters infected with 50 or 100 metacercariae (Mc) showed higher antibody levels than those infected with 25 Mc. Then, the antibody level increased rapidly to a plateau at approximately 2 mo. The antibody levels to egg and somatic, but not to Es antigens, were significantly higher in the hamsters infected with 25 Mc than in those

infected with 50 or 100 Mc. These antibody responses, particularly to egg and Es antigens, did not correlate with worm burden or egg output[59]. Secretory IgA antibody reacted with both egg and Es antigens also appeared in the bile of infected hamsters. IgG, IgA and IgE antibodies in serum and bile have been reported in patients with opisthorchiasis[61-64]. Analysis of bile and serum from individual patients showed that while IgG was the predominant in the serum of all patients, IgA was present at approximately the same level as IgG or higher in the bile, while IgE antibody could be detected in 50% of the bile samples[64]. The IgG levels to the somatic antigen were most markedly elevated in disease cases and were closely associated with gall bladder size and dysfunction, and also significantly correlated with opisthorchis egg counts[32]. The antibody response to *O viverrini* decreased after treatment[65,66]. Immunoblot analysis revealed a 91/93-ku metacercaria homogenate doublet which was recognized only in sera of individuals with severe liver disease on ultrasound findings, including cholangiocarcinoma[64,67,68].

Cell-mediated immune responses (CMIR) to *O viverrini* infection have been demonstrated. Granulomatous inflamatory response to adult flukes and eggs during chronic infection is one of the typical CMIR. The inflammatory granuloma is surrounded by eosinophilic precipitates, neutrophils, eosinophils, mononuclear cells and macrophages after 6 wk of infection[24]. Similarly, patients infected with *C sinensis* exhibited egg-induced granuloma, both in the gall bladder and in the liver portal area[69,70]. Although both cellular and humoral immunity to *O viverrini* have been reported, there exists a striking difference in the antigenic recognition pattern of T and B cells which may be important for selecting antigen molecules for immunological studies of this trematode infection in man[71]. The molecular weight of the main immunogenic fractions for T cells ranged from 28 to 46 ku, whereas those recognized by antibodies were 45, 52, 56, 59, 65, 69, 75 and 81 ku. The exposure to *O viverrini* depressed the egg and egg reduction could be due to biliary obstruction and not necessarily to immunological damage of the reproductive system of the flukes. Biliary obstruction is unlikely to be the major factor as it is known to be uncommon in experimental opisthorchiasis[24,72]. Infection of hamsters with *O viverrini*, whether or not eliminated with praziquantel before the time of the re-challenge, failed to confer any protective immunity to reinfection[73].

An attempt to potentiate acquired immunity in hamsters with prior infection by immunizing them with aqueous somatic extract of adult worms resulted in a 30% reduction of worm[74]. IgG, IgA and IgM were found significantly higher levels to adult worm homogenate and metacercaria homogenate in stool of the egg-negative residents in the *O viverrini*-endemic area comparing to stool of the egg-positive group, thereby indicating some protective evidence of opisthorchiasis[75]. The lack of protective immunity is perhaps due to suppression of both cellular and humoral immune responses to unrelated antigens by *O viverrini* as shown by reduced phytohemagglutinin-induced lymphoproliferation and response to sheep red blood cell stimulation[68,75]. Moreover, when specific antibody was measured in the infected animals, the titer was found to be depressed during the late stage of infection, particularly in heavily infected group[77]. The parasites may be resistant to immune damage or able to evade the defense system of the host. The inability to kill the worms by serum from infected animals or from patients with opisthorchiasis, and the resistance to immune damage may be related to tegumental shedding and repair[68,76]. The role of T cells and cytokines in protective immunity and pathogenesis of opisthorchiasis is also unclear. The set of cytokines produced by these different T helper responses, in turn, can exert opposing effects on the parasite, resulting in either control of infection or promotion of disease. *O viverrini* may induce the Th2 response as found with other flukes, such as *Schistosoma mansoni*. However, information regarding the significance of immune responses generated during liver fluke infections is limited[68].

Diagnosis of O viverrini infection

Diagnosis of the infection is basically based on the standard fecal examination of the fluke's eggs. However, in cases with bile duct obstruction or in a light infection, eggs may not be detected in the feces[77]. Detection of fecal *Opisthorchis* antigen has been reported[78,79], though it is not so convenient. There is a need for a reliable diagnostic tool to solve the clinical and parasitological diagnosis problems in opisthorchiasis. Immunodiagnostic method is one of highly sensitive techniques in diagnosis of *O viverrini* infection. Several serologic tests for the diagnosis of opisthorchiasis have been reported[58,65,78-83]. However, some cross-reactions with other parasitic infection are still a problem. The recombinant cysteine proteinase-based ELISA was used to diagnose the infected hamsters and humans. The use of *Ov*-CP-1 as antigens showed that humoral immune responses in hamsters infected with 50 Metaceriae of *O viverrini* occured as early as d 15 post-infection and rapidly increased at one month before reaching a plateau at mo 2, which is in agreement with that reported by using *O viverrini*-somatic extract, excretory-secretory product or egg antigens[58]. In human cases, the *Ov*-CP-1-based ELISA did not cross-react with sera from patients with hookworm, minute intestinal fluke, *S stercoralis, Taenia sp., G lamblia* and *E. coli* infection. The sensitivity and specificity of the test were 94.66% and 95.55%, respectively. This is even higher than conventional indirect ELISA using *O viverrini* somatic extracts or excretory-secretory product antigen[63,64,79,81]. The sensitivity and specificity are similar to other studies using ELISA based on recombinant trematode cysteine protease, such as *C sinensis* (sensitivity: 81.3%-96%; specificity: 92.6%-96.2%)[84,85] and *P. westermani* (sensitivity: 86.2%-93%; specificity: 93%-98%)[86,87]. It seems that the more specific purified antigen used, the more sensitivity and specificity obtained. Recently, Ruangsittichai *et al*[54] reported a high sensitivity and specificity of a recombinant eggshell protein (ESP) as a potential serodiagnosis of human opisthorchiasis. However, detection of *O viverrini* DNA is expensive and requires skillful professional personnel. This overall result suggests that *Ov*-CP-1 has potential as an antigen for ELISA-based immunodiagnosis of opisthorchiasis.

PCR-based amplification of parasite DNA from stools has been documented for its high specificity. Compared with that of formalin-ether and Stoll's egg count, the sensitivity of the PCR was found to be 100%, 68.2%, and 50% in the fecal specimens containing > 1000, 200-1000, and < 200 eggs per gram of feces, respectively. Human fecal materials showed a strong reduction in PCR sensitivity of detection[51,52]. Recently, an adult stage *O viverrini* cDNA library was constructed and was isolated a glutathione S-transferase (GST) cDNA. The cDNA has a size of 893 bp and encodes a GST of 213 amino acids (OV28GST). The OV28GST RNA was detected in the parenchymal cells of adult parasites. Both purified recombinant and purified native OV28GST were resolved as 28-ku proteins. Molecular analysis of OV28GST is helpful in ongoing development of diagnostic applications for *O viverrini*[54].

O viverrini is a medically important food-borne trematode in some part of Southeast Asian countries, including Thailand. The opisthorchiasis has been studied for more than 50 years, a major break through in pathology and pathogenesis of opisthorchiasis was the discovery of *Opisthorchis* antigens in the biliary epithelium and its associated intense inflammatory response in certain infected animals[25]. The infection is associated with cholangiocarcinoma and other hepatobiliary diseases. The epidemiology is found in some rural areas because of the tradition of eating raw or uncooked fish products. The health education programs to prevent and control opisthorchiasis are still required in the high-risk areas.

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REVIEW

Severe acute pancreatitis: Pathogenetic aspects and prognostic factors

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Abstract

Approximately 20% of patients with acute pancreatitis develop a severe disease associated with complications and high risk of mortality. The purpose of this study is to review pathogenesis and prognostic factors of severe acute pancreatitis (SAP). An extensive medline search was undertaken with focusing on pathogenesis, complications and prognostic evaluation of SAP. Cytokines and other inflammatory markers play a major role in the pathogenesis and course of SAP and can be used as prognostic markers in its early phase. Other markers such as simple prognostic scores have been found to be as effective as multifactorial scoring systems (MFSS) at 48 h with the advantage of simplicity, efficacy, low cost, accuracy and early prediction of SAP. Recently, several laboratory markers including hematocrit, blood urea nitrogen (BUN), creatinine, matrix metalloproteinase-9 (MMP-9) and serum amyloid A (SAA) have been used as early predictors of severity within the first 24 h. The last few years have witnessed a tremendous progress in understanding the pathogenesis and predicting the outcome of SAP. In this review we classified the prognostic markers into predictors of severity, pancreatic necrosis (PN), infected PN (IPN) and mortality.

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Key words: Acute pancreatitis; Pathogenesis; Prediction; Severity; Necrosis; Infected necrosis; Mortality

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INTRODUCTION

Acute pancreatitis (AP) remains a serious disease. It is defined as an inflammatory process of the pancreas with possible peripancreatic tissue and multi-organ involvement inducing multi-organ dysfunction syndrome (MODS) with an increased mortality rate[1]. The majority of patients present with a mild disease, however approximately 20% run a severe course and require appropriate management in an intensive care unit. According to the Atlanta classification, severe acute pancreatitis (SAP) is defined as an AP associated with local and/or systemic complications. Atlanta classification is a clinically-based classification defining AP, severity and complications. This classification has been proposed in the international symposium on AP, Atlanta, 1992 by 40 international authorities from 15 countries and six medical disciplines[1]. Development of organ dysfunction within 72 h of symptom onset is defined as early severe acute pancreatitis (ESAP). Early severe acute pancreatitis is characterized by a short course, progressive MODS, early hypoxemia, high computed tomography (CT) score, increased incidence of necrosis infection and abdominal compartment syndrome (ACS)[2,3].

Multi-organ dysfunction syndrome, the extent of pancreatic necrosis, infection and sepsis are the major determinants of mortality in AP[4-6]. Pancreatic necrosis is considered as a potential risk for infection, which represents the primary cause of late mortality. Occurrence of acute respiratory (ARF), cardiovascular (CVF) and renal failures (RF) can predict the fetal outcome in SAP[7]. A wide range of mortality (20%-60%) has been reported in SAP[8,9]. Early diagnosis and prognostic evaluation are extremely important and may reduce the morbidity and mortality associated with SAP.

PATHOGENESIS

Inappropriate intracellular activation of proteolytic enzymes induces pancreatic tissue damage in the presence of intracellular lysozyme enzymes and activation of trypsinogen to trypsin. Trypsin in turn activates a cascade of phospholipases, elastases and other mediators with increased neutrophil migration to the pancreas. Consequently a variety of inflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), platelets activating factor (PAF) and tumor necrosis factor (TNF) are released[10]. Apparently proinflammatory cytokines play a pivotal role in the pathogenesis of AP and its systemic complications[11]. IL-1 and TNF induce the release of IL-6 and IL-8[12,13] The presence of high C-reactive protein (CRP) and serum amyloid A (SAA) strongly suggests their major role in

the pathogenesis of AP[14]. Metalloproteinase-9 (MMP-9) may alter the permeability by disruption of the basement membranes of the vascular endothelial barrier[15,16]. Additionally, in experimental pancreatitis activation of mast cells has contributed to the intra and extrapancreatic endothelial barrier dysfunction and may partly contribute to the development of MODS[17]. The endothelial barrier damage in various organs including the pancreas, lungs, kidneys and intestine is associated with an increased permeability and consequent passage of blood constituents to various tissues[18-20]. Lung involvement with development of pleura effusion indicates systemic involvement with an increased mortality risk[21]. In addition, inflammatory mediators may also affect intestinal microcirculation leading to damage of microvilli allowing endotoxine and bacterial translocation (BT) into the systemic circulation and hence increase the risk of pancreatic infection[22,23]. Hyperstimulation of the immune system determines the immunosuppression and the increased mortality risk in the course of acute necrotizing pancreatitis. In the early stage of AP, cytokinemia and the oxidative stress are responsible for the inadequate immune response[24,25]. The inflammatory process is associated with acinar cell damage and an inadequate immune response mediated mainly by monocytes, macrophages, polymorphonuclear cells (PMNs), lymphocytes, mast cells and endothelial cells[26,27]. In SAP the inflammatory process is amplified by the release of cytokines and reactive oxygen species (ROS)[28]. The intensity of oxidative stress correlates well with the severity of AP[24,25]. Antioxidants such as glutathione peroxidase (GPx) play a pivotal role in the process of defense especially, where ROS contribute significantly to the disease etiology[29,30]. Glutathione peroxidase activity is selenium-dependent and is lowered in states associated with selenium deficiency[28]. In the presence of cytokines and ROS, nuclear factor-κB (NF-κB) is activated. It links the inflammatory response and regulates several forms of proinflammatory gene expressions including TNF-α, IL-1, IL-8, chemokines, selectin-E and intracellular adhesion molecule-1 (ICAM-1)[31,32].

During infection, the liver produces large amounts of SAA and other inflammatory markers. Serum amyloid A plays an important role in the activation of PMNs and chemotaxis of PMNs, monocytes and T-lymphocytes and release of proinflammatory mediators such as TNF-α, IL-b and IL-8 from PMNs[33,34]. Neutrophils are also activated by MMP-9, which promotes their endothelial cell adhesion and migration into the inflamed tissue[35]. Proteolytic activity of MMP-9 may further mediate pathological inflammation[36]. TNF-α, IL-1b and IL-8 can stimulate the release of MMP-9, which damage basement membranes and alter permeability[15,16].

Several recent studies have emphasized the role of genetic polymorphism in the predisposition to immune response variations and severe clinical course of AP. This supports the knowledge of genetic and environmental factors as severity modifiers of the disease and helps to provide prophylactic and therapeutic strategies that minimize the morbidity and mortality of SAP. For instance, monocyte chemotactic protein-1 (MCP-1) is increased in the early course of AP and can therefore accurately predict the severity and mortality[37]. Oxidative stress may also contribute to the pathogenesis of SAP. Glutathione-s-transferase (GST) genetic polymorphism also enhances the inflammatory response to pancreatic injury and promotes the progression of mild acute pancreatitis (MAP) to SAP due to the oxidative stress-induced glutathione depletion[38]. In contrast, TNF-α polymorphism has had no relation to severity of pancreatitis, but there has been a strong association with severity of sepsis complicating SAP[39]. However, in another study TNF-α and heat shock protein (HSP-70-2) polymorphism has been associated with risk of SAP[40].

Pancreatic necrosis (PN)

Approximately 20% of patients with AP develop PN. Various factors including ischemia, lysozyme enzymes, vasoactive mediators and ROS are responsible for the development of PN. It is assumed that the lysozomal protease, that activates trypsinogen in the presence of intracinar colocalisation of digestive and lysozyme enzymes, is responsible for the acinar necrosis[41]. Other vasoactive mediators that contribute to the development of PN include endothelin-1[42], phospholipase A2 (PL-A2)[43] and bradykinin inhibition[44]. Phospholipase-A2 also mediates the development of PN and strongly correlates with severity of AP as well as with occurrence of pulmonary and renal complications. Severe AP is associated with microvasculature damage, compromise of the microcirculation and release of ROS from leukocytes and macrophages[45]. Chronic alcohol consumption constitutes a strong risk factor for PN as has been shown in a recent two-arm study[46]. Alcohol, whether acute intake or chronic consumption, increases monocyte response to inflammatory signals and may enhance the inflammatory process in pancreatitis[47]. It is assumed that the most significant determinant prognostic factor of AP is development of PN with subsequent complications[48]. Acute necrotizing pancreatitis consists of an early phase during the first two weeks characterized by a sterile PN and systemic inflammatory response syndrome (SIRS) and a second phase with infection of the PN, septic complications and MODS[49].

Infected pancreatic necrosis (IPN)

The presence of bacteria in focal or diffuse areas of intra- or extrapancreatic tissue is defined according to the Atlanta definition as IPN[1]. The gut represents the main source of pancreatic contamination and related septic complications[49]. The mechanism of pancreatic infection is not yet fully explored. However, recent investigations have focused on the bacterial translocation (BT) from the intestine. Systemic inflammatory response syndrome associated ischemia, release of ROS and acidification are responsible for the mucosal dysfunction in patients with SAP[50]. Transmural[51], hematogenous[52] and lymphogenous[53] spread are the major pathways of BT. Several factors including altered gut motility, immunosuppression, bacterial overgrowth and damaged mucosal barrier promote BT and hence pancreatic contamination with septic complications and increased mortality rate[54]. Acute pancreatitis by itself

may promote BT with subsequent pancreatic infection and related complications[55]. Experimental studies have suggested BT *via* a damaged intestinal barrier[45] and immunosuppression[56] as the two important key factors of pancreatic contamination.

The incidence of infection in AP usually does not exceed 10%[57], however it approaches 80% in NP with increased morbidity and mortality rates[58,59]. The risk of infection is correlated with the extension of necrosis. It occurs in 30%-40% of patients with ⩾ 30% necrosis. Aggravation of abdominal pain, fever, leukocytosis, although not specific, may indicate infection of pancreatic necrosis. However, a definite diagnosis is established only by US- or CT-guided fine needle aspiration (FNA)[60,61]. Currently, FNA and culture of the aspirate are recommended to confirm IPN in patients with sepsis[62]. The mortality rate in IPN is around 25% compared to 10% in sterile necrosis and no mortality in MAP[63]. Infection and septic complications account for 60%-80% of mortality in AP[64,65].

Multi-organ dysfunction syndrome (MODS)

Several factors are thought to contribute through their injurious effect on the endothelial barrier to the pathogenesis of MODS. These factors include cytokines[66], ROS[67], PL-A2, elastases[68] and calcium flux[69]. In SAP the development of MODS is a result of hemodynamic abnormalities possibly secondary to activation of kinin and other vasoactive peptides[70]. Reactive oxygen species originate from various sources including xanthin oxidases, mitochondrial cytochrome oxidases, arachidonic acid metabolism and PMNs[71,72]. In SAP, the microcirculatory dysfunction is apparently mediated by ROS, leukotriens, PAF, interleukins and other proinflammatory mediators[73]. Proinflammatory cytokines (TNF-α, IL-1b and IL-6) play a significant role in the pathogenesis of AP and may mediate systemic complications[11]. These complications may result from an exaggerated and uncontrollable host response leading to SIRS[74]. Multi-organ dysfunction syndrome is apparently induced by cytokines, and inflammatory mediators released in a state of SIRS[2,75] which occurs in 72%-90% of patients with SAP. Single organ failure (SOF) accounts for 25%-37% and multi-organ failure (MOF) accounts for 35%-66% of MODS. The most commonly encountered SOF has been respiratory, cardiovascular, hepatic and renal failure in 39.1%-63%, 23%-37.7%, 20.7% and 5.8%-13% of patients, respectively[76-78]. In a similar study, 61.7% have had MOF and 26.6 SOF mainly respiratory (35.1%), cardiovascular (22.3%), GI (19.1%), hepatic (15.9%) and renal (14.9%) in origin[7]. Development of MODS is considered a major risk of mortality[11,79].

Abdominal compartment syndrome (ACS)

The combination of intra-abdominal hypertension (IAHT) and end-organ dysfunction has been described as ACS. It is determined by measurement of abdominal pressure[80]. The diagnosis of ACS is established when intra-abdominal pressure exceeds 15 mmHg (2 kPa) associated with low cardiac output, progressive oliguria and hypoxia[2]. Abdominal compartment syndrome is associated with a high mortality rate correlated with sepsis and MODS. Recent reports have described the association of ACS with SAP. Approximately 11% of patients with SAP develop ACS with a higher incidence in fulminant AP, higher mortality[81-83] and organ failure rates in the majority of patients[84]. More frequently, IAHT has occurred in association with ESAP. Approximately 78% of patients with ESAP have had IAHT[2]. Abdominal compartment syndrome may develop in association with an aggressive fluid replacement[85] and during the infection and SIRS stages[86]. It is mainly correlated with uncontrolled and sustained IAHT and characterized by a severely distended abdomen and MODS. Anuria and IAHT are the most important determinants of mortality[87].

Mortality

Several authors have reported various rates of mortality varying according to the stage and severity of AP. While MAP has no mortality, the rate amounts to 10% in NP and 25% in IPN[63]. Higher rates ranging from 20%-60%[8,9] and 30%-90% in fulminant pancreatitis[88] have been reported. There are two peaks of mortality in SAP. The early phase mortality, mostly due to SIRS and MODS, occurs within the first few days and the second peak induced by PN, IPN and MODS occurs after the second week[79]. Approximately 23% of the early mortality has occurred within the first 3 d and 53% within the first week[3]. Another study has reported early mortality in 51% within the first two weeks primarily due to MODS and later 49% mainly due to IPN[89]. Sepsis and MODS may complicate pancreatic contamination contributing to > 80% of mortality[65]. Early diagnosis and appropriate management are important to decrease morbidity and mortality. A delay of transfer to ICU > 24 h after admission is associated with a four fold increased mortality risk[90]. Several factors such as etiology, age, gender, race, genetic makeup, severity on admission, the extent and infection of PN have been reported to influence the mortality[77]. On the contrary, other authors have reported a correlation between the mortality and the severity of the attack but no correlation with the cause of AP[88]. The persistence of organ failure is a strong marker of poor outcome and mortality[91]. PN, IPN and MODS are the major determinants of mortality[6,58]. The majority of deaths are due to MODS[6,92]. In a multivariate logistic analysis ARF,CVF and RF have been the independent prognostic factors of mortality[7] and IPN and MODS are the most significant causes of hospital mortality[93,94]. Other authors have reported ARF and RF as the main cause of mortality and no influence of PN on mortality in SAP[95]. Despite improvement in diagnostic and therapeutic modalities, SAP remains associated with a high mortality rate[96] ranging from 15%-20%[88] and may be as high as 40%[97]. While some authors have reported a persistence of the mortality rate for the last decades[96,98], others have reported a significant decline[79,99].

PROGNOSTIC EVALUATION

SAP is associated with a high early mortality rate (30%-50%) mostly as a result of MODS[100,101]; hence early diagnosis and appropriate management are essential. However, identification of severe cases during the first

Table 1 Predictors of severity of acute pancreatitis

Severity predictor	Reference
Multifactorial scoring system	
Ranson, Imrie, APACHE II	[14]
Clinical data	
Age	[108]
Etiology	[108]
Obesity	[63,109]
Simple prognostic scores	
BUN (=/> 25 mg/dL), LDH (=/> 900 IU/L) and PN on CE-CT	[104]
Age, highest serum creatinine (within 72 h), chronic health status and requirement for mechanical ventilation	[119]
Inflammatory markers:	
IL-6, IL-8	[10]
PL-A2, CRP	[64]
PMN-elastase	[122]
TAP	[123]
PCT	[124]
CT-scoring index (CTSI)	[140]

PN: Pancreatic necrosis; CE-CT: Contrast enhanced computed tomography; IL-6: Interleukin-6; IL-8: Interleukin-8; PL-A2: Pancreatic phospholipase A2; TAP: Trypsinogen activation peptide; CRP: C-rective protein; PTC: procalcitonin; PMN: Polymorphonuclear cells; LDH: Lactate dehydrogenase.

Table 2 Early predictors of severity

Marker		Period	Reference
Clinical:	Obesity	on admission	[111,112]
Radiological:	Abnormal chest radiograph	within 24 h	[21]
Laboratory:	Serum BUN	within the 1st 24 h	[104]
	Serum creatinine	within the 1st 24 h	[21]
	Hematocrit	on admission	[133]
	Proteinuria	2nd d of admission	[111]
	CRP	24-48 h	[129]
	MMP-9	1-48 h of admission	[132]
	SAA	within the 1st 24 h	[43]
	IL-6, IL-8	within the 1st 24 h	[10]
	IL-18	within the 1st 48 h	[28]
	Trypsinogen-2	on admission	[131]
	Trypsin-2 AAT	on admission	[131]
Factors (in biliary pancreatitis) predicting need for ICU:			
	Heart rate, Glucose, BUN, WBC&CRP	on admission	[136]

CRP: C-reactive protein, MMP-9: Matrix metalloproteinase-9; SAA: Serum amyloid A; IL-6, 8, 18: Interleukins 6, 8 and 18; BUN: Blood urea nitrogen; WBC: White blood cell count, trypsin-2; AAT: Trypsin-2-alpha-1-antitrypsin.

2-3 d of symptoms onset, where MODS takes place, is difficult[102,103]. The best predictors include clinical features, markers of pancreatic injuries and markers of inflammatory response[63].

Currently, several markers are used for prognostic evaluation of different stages in the course of AP and development of complications. They can be classified as markers of severity, early prediction, PN, IPN and mortality (Tables 1-5).

Markers of severity

Multifactorial scoring systems (MFSS): All MFSS such as Ranson, Glascow, Imrie, APATCHE Ⅱ and clinicobiochemical systems have a similar accuracy after 48 h of admission[14]. They are good predictors of severity and PN[104]; however they have insignificant value for prediction of infection in patients with SIRS[105,106]. MFSS are complex, consist of several criteria[104] and are less accurate than the calcitonin precursor in the prediction of infection in patients with SIRS[107] and consume 48 h.

Table 3 Predictors of pancreatic necrosis

Marker	Reference
1 C-reactive protein (CRP)	[48,148]
2 Serum lactate dehydrogenase (LDH)	[153]
3 Procalcitonin (PCT)	[151]
4 Serum amyloid A (SAA)	[14]
5 Serum macrophage migration inhibitory factor (MIF)	[152]
6 Contrast enhanced CT (CE-CT)	[153]

Table 4 Markers of infected pancreatic necrosis

Marker	Reference
1 C-reactive protein (CRP)	[64]
2 Procalcitonin (PCT)	[64,124]
3 Serum amyloid A (SAA)	[14]
4 Interleukin-6, 8 (IL-6, IL-8)	[64]
5 Soluble intracellular adhesion molecule (sICAM-1)	[58]
6 Reactive nitrogen intermediates (RNIs)	[155]
7 US- or CT-guided FNA	[14,60,61]

Table 5 Markers of mortality

Marker	Reference
1 Obesity	[111,112]
2 Serum creatinine > 2 mg/dL	[21]
3 Pleura effusion on chest radiograph	[21]
4 Early nonenhanced CT	[157]
5 Multiorgan dysfunction syndrome (MODS)	[6,92]
6 Pancreatic necrosis (PN)	[6,92]
7 Infected pancreatic necrosis (IPN)	[6,92]
8 Interleukin-6 (IL-6)	[90]
9 Reactive nitrogen intermediates (RNIs)	[58,156]

Clinical data: Some recent reports have discussed the predictive value of clinical data such as age, etiology[108] and obesity[63,109]. Obese individuals have had a worse prognostic score on admission and have developed significantly more complications compared to non-obese patients. For instance, respiratory failure has occurred more frequently in obese subjects[109]. Also the rates of infective complications such as IPN, sepsis and pancreatic abscess have been higher in obese patients[109,110]. Generally, obesity is more frequently associated with SAP, local and systemic complications and mortality; hence it should be used routinely in the initial assessment of severity[111,112]. Obesity increases the risk for SAP by enhancing the immune response to pancreatic injury[113]. Body mass index (BMI) > 30 kg/m^2 is a reliable predictor of severity[63,114]. However, another study has found no effect of BMI on pro- or anti-inflammatory cytokines levels in early pancreatitis[115].

While some studies have not found a correlation between etiology on one hand, course and outcome of AP on the other hand[88,116], others have reported the highest mortality rate in patients with idiopathic pancreatitis[117,118].

Laboratory markers: Current prognostic markers of severity are valuable, but immediate evaluation is difficult to establish[109]. However, Simple prognostic score (SPS), consisting of blood urea nitrogen (BUN) ⩾ 25 g/dL, serum lactate dehydrogenase (LDH) ⩾ 900 U/L and presence of PN on contrast enhanced-CT (CE-CT), is comparable with MFSS with the advantage of simple prediction of SAP on admission[104]. Another SPS constituting four variables including age, highest serum creatinine within three days of primary admission, chronic health status and mechanical ventilation requirement have had a similar predictive performance as APACHE Ⅱ[119].

Inflammatory markers: Cytokines have a central role in the pathogenesis of SAP. The proinflammatory cytokine response is reflected by an increased IL-6 level and release of anti-inflammatory mediators. Elevated levels of pro- and anti-inflammatory cytokines occur early, persist for several days in systemic circulation and are independent of sepsis. By univariate analysis an IL-6 level > 1000 pg/mL is associated with an increased risk of mortality at day one[90]. Inflammatory markers such as IL-6, TNF-α, PL-A2 and CRP are good predictors of severity, however, their role in identifying those at risk to develop IPN is not determined yet[64]. Significantly higher levels of IL-6, IL-8 and TNF-α differentiate independently MAP from SAP. TNF-α is difficult to measure compared to IL-6 and IL-8[10]. IL-ra and IL-15 are useful predictors of complications, especially MODS[120,121] and mortality[121].

Other markers such as PMN-elastase[122], trypsinogen activation peptide (TAP)[123] and procalcitonin (PCT)[124] are elevated in SAP and can be used for severity assessment. They are able to predict the severity, but they are not widely available. Currently, CRP is the only marker applied routinely in clinical use[125]. It is considered as the standard severity and prognostic serum marker in SAP[126]. The levels of CRP in SAP have differed from control and MAP after 48 h of symptom onset. In contrast, SAA plasma level has differed significantly ($P < 0.001$) in SAP from MAP and control and can predict severity earlier and better than CRP[14]. In SAP CRP and SAA levels are significantly higher than other acute phase proteins[127].

Early markers for prediction of severity

Various markers including IL-6, IL-8[119,128], TNF-α, PMN-elastase[129], TAP[130], PLA-2[43] can be used as early predictors of SAP, however they need to be made clinically applicable[63]. CRP is the widely used and clinically applied marker of severity 48 h after the onset of symptoms[129]. Other early predictors of severity include a marked decrease of GPx and selenium serum concentration, which reflect the significance of oxidative stress, are associated with a marked increase of IL-18 during the first two days of admission in patients with SAP. Hence, GPx and IL-18 may be used as predictors of severity[28]. Trypsinogen-2 and trypsin-2-alpha-1-antitrypsin (trypsin-2 AAT) have been reported as early accurate markers of severity already at admission, therefore, they are considered as useful markers for clinical use[131].

In addition, MMP-9, a Zn^{2+} containing enzyme[132] has been reported as a valuable early predictor of severity. It significantly degrades the basement membrane components in the inflammatory process and is involved in the deterioration of SAP. In comparison to normal subjects and those with MAP, it increases significantly one hour after admission, decreases after 48 h and is able to differentiate SAP from MAP already one hour after admission. It also correlates well with CRP and TNF-α levels and APACHE Ⅱ in patients with SAP[98].

BUN, a marker of hypovolemia and catabolism has been suggested as the most useful prognostic predictor in the first 24 h of SAP onset[104]. In addition, serum creatinine level exceeding 2 mg/dL (> 176.8 μmol/L) and the presence of pleura effusion or parenchymal densification on chest radiography identify within 24 h of admission those with a severe or adverse clinical course[21]. The presence of pleural effusion particularly, left-sided or bilateral, has an excellent predictive value[99]. Furthermore, patients with SAP commonly develop proteinuria, which can discriminate on the second day of admission SAP from MAP[111].

Hematocrit (Hct) has also been reported as an early, reliable, easily obtainable and cheap marker. Its prognostic value on admission is comparable to MFSS after 48 h of admission. It limits the requirement of CE-CT to those who do not improve[133]. On the contrary, other authors have not found a significant difference in the decrease of the 24 h Hct concentration in MAP and SAP[134] and hemoconcentration has recently been reported as a poor predictor of PN[135]. In patients with biliary pancreatitis serum glucose, BUN, WBC, tachycardia and APACHE Ⅱ scores independently predict patients requiring intensive care management[136].

Early localization of PN by CE-CT can predict the outcome in SAP. Necrosis of the head or the entire pancreas is associated with high risk complications compared to the distal part[137]. The accuracy is enhanced by additional use of Hong-Kong simple biochemical criteria (BUN and glucose)[138]. Additionally the presence of extrapancreatic collection and non-visualization of portal and splenic veins on CT predicts complicated outcome in SAP[139]. Balthazar CT-severity index (CTSI) ⩾ 5 is an accurate tool for assessing the severity and outcome of AP. It has been superior to Ranson's criteria and APACHE Ⅱ in predicting outcome of AP[140].

Lipid peroxidation products, which suggest a pivotal role of oxidative burst, may help to predict the prognosis in SAP[141]. Also plasma alph2-macroglobulin proteolysis, which induces local and distant tissue injuries and has been associated with increased MMP-2, MMP-9 and serine proteinase plasma levels can be used as a prognostic marker of severity in SAP[142]. Further, recently reported prognostic markers include SAA and neopterin. Serum amyloid A is a sensitive marker of inflammation and tissue injury. It predicts early severity and it has been superior to CRP within the first 24 h of symptom onset[43]. Neopterin, a marker of macrophage activation along with IL-6 serum

levels reflect pancreatitis severity and therefore it may act as a predictor of severity within the first week of AP onset[143]. While PCT has been reported to be of limited additional clinical value in the early assessment of AP[144], it has been found to be an early predictor of severity and can be used to monitor the clinical prognosis[145]. In addition, a more recent prospective international multicenter study has reported PCT as an early and reliable marker of clinically relevant pancreatic infections and the overall prognosis in SAP[146]. The initial data of a recent study has also suggested serum proteomic profile as a predictor of severity that differentiates SAP from MAP[147].

Markers of PN

PN usually develops within the first three days of SAP onset. Several markers are implicated in the prognostic evaluation of PN. These include IL-6[78], TNF[134], CRP, PMN-elastase[148], TAP[149] and SAA[14]. Among these markers CRP is the only available marker in clinical laboratories[59]. CRP is produced in the liver cells under stimulation by IL-1 and IL-6. Although the peak serum concentration is reached after 72 h[150], it is able to differentiate SAP from interstitial pancreatitis within 24 h. CRP with a cut-off level of 110 mg/L has a highest sensitivity and negative predictive value for PN[48]. Its predictive value for PN has been higher than those of PCT and IL-8. The area under the curve (AUC) of ROC has been 0.92, 0.79 and 0.52 respectively[151]. CRP concentration rises significantly in early stages of PN with a high sensitivity and negative predictive value (NPV) of approximately 95%. In a multivariate analysis serum macrophage migration inhibitory factor (MIF) has recently been reported to be an independent predictor of PN[152]. CE-CT is a valuable diagnostic modality for PN with almost 100% sensitivity between 4 and 10 d. It is the gold standard method that confirms the diagnosis of PN; however CRP and LDH, a marker of tissue damage, are routinely utilized as markers of PN[153].

Markers of IPN

The incidence of IPN is influenced by the extent of necrosis and is associated with a prolonged hospital stay and increased rates of morbidity[154] and mortality[155]. In the early stages of the disease there has been no significant difference in the median CRP concentration between IPN and sterile pancreatic necrosis (SPN). Univariate and multivariate regression analysis have shown a correlation between CRP concentration and secondary pancreatic contamination and therefore it has been suggested as a marker to differentiate preoperatively IPN from SPN[64]. Procalcitonin serum level is markedly higher in IPN compared to SPN during the first week. Compared to CRP and IL-6, PCT at a cut-off value of 1.8 ng/mL is closely correlated with the presence and intensity of necrosis infection. On the other hand, a combination of PCT < 2 ng/L and IL-6 < 400 pg/L has been the best to identify patients not at risk of secondary pancreatic contamination. PCT is suggested as a potential and accurate new marker for prediction of IPN[64]. It is a nonspecific marker of infection and sepsis, posses the same diagnostic accuracy of FNA and it correlates well with the presence and severity of infection[64,124].

IL-6 is increased for several days in both infected as well as sterile necrosis, however with significantly higher levels in IPN. Also IL-8 is elevated for a few days in both types of necrosis with markedly higher levels in IPN[64].

IL-8, a potent PMN activating cytokine, is a good indicator of septic MODS in patients with SAP. Other inflammatory cytokine levels for instance, sICAM[58] and nitric oxide in the form of reactive nitrogen intermediates (RNIs)[155] are elevated in both sterile and infected necrosis. Higher levels of NO are associated with an increased risk of sepsis and mortality[156].The gold standard for an accurate and early diagnosis of IPN is the US- or CT-guided FNA with an approximately 90% sensitivity and specifity[14].

Predictors of mortality

Recently, nonenhanced CT has been found to be a valuable predictor of mortality[157]. A novel model for prediction of mortality consisting of four variables: age, highest serum creatinine level within 60-72 h from primary admission, requirement for mechanical ventilation and chronic health status with at least a similar predictive performance of APACHE Ⅱ can predict fetal outcome in the early stage of SAP[119]. ARF, CVF and RF predict mortality in patients with SAP[7]. Systemic complications especially ARF and RF are the main causes of mortality in SAP[95]. Also the presence of chronic renal failure (CRF) worsens the prognosis of AP and is associated with high morbidity and mortality[158]. In a univariate analysis, an IL-6 concentration > 1000 pg/mL has been associated with an increased risk of the first 24 h mortality[90]. Also IL-ra and IL-15 are useful predictors of mortality[120,121].

The mortality risk is correlated with higher serum NO concentration[58]. A serum creatinine level > 2 mg/dL and an abnormal chest radiograph together and isolated are significantly associated with a high mortality risk at 90% sensitivity and 76% specifity[21].

The persistence of organ failure is a strong marker of poor outcome and mortality[91]. PN, IPN and MODS are the major determinants of mortality[6,92].

CONCLUSION

The intracellular presence of inappropriately activated proteolytic enzymes is responsible for the damage of pancreatic tissue. Trypsin in turn activates a cascade of inflammatory markers, which are together with ROS responsible for the development of acute pancreatitis and its complications. Early diagnosis and prognostic evaluation may reduce morbidity and mortality. Prognostic predictors of severity, PN, IPN and mortality are summarized: (1) early severity predictors: obesity, abnormal chest radiograph, serum BUN, creatinine, proteinuria, hematocrit, CRP, MMP-9, SAA, IL-6, IL-8, and IL-18, trypsinogen-2 and trypsinogen-2 AAT, (2) prognostic predictors of necrosis include CE-CT, LDH, CRP, PCT, IL-6, SAA and MIF, (3) prognostic predictors of IPN: CRP, PCT and ultrasound or CT-guided FNA, (4) prognostic predictors of mortality: obesity, pleural effusion on chest radiograph, IL-6, RNIs, PN, IPN, and MODS.

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TOPIC HIGHLIGHT

Wei Tang, MD, EngD, Assistant Professor, *Series Editor*

Surgical treatment of hepatocellular carcinoma: Evidence-based outcomes

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Abstract

Surgeons may be severely criticized from the perspective of evidence-based medicine because the majority of surgical publications appear not to be convincing. In the top nine surgical journals in 1996, half of the 175 publications refer to pilot studies lacking a control group, 18% to animal experiments, and only 5% to randomized controlled trials (RCT). There are five levels of clinical evidence: level 1 (randomized controlled trial), level 2 (prospective concurrent cohort study), level 3 (retrospective historical cohort study), level 4 (pre-post study), and level 5 (case report). Recently, a Japanese evidence-based guideline for the surgical treatment of hepatocellular carcinoma (HCC) was made by a committee (Chairman, Professor Makuuchi and five members). We searched the literature using the Medline Dialog System with four keywords: HCC, surgery, English papers, in the last 20 years. A total of 915 publications were identified systematically reviewed. At the first selection (in which surgery-dominant papers were selected), 478 papers survived. In the second selection (clearly concluded papers), 181 papers survived. In the final selection (clinically significant papers), 100 papers survived. The evidence level of the 100 surviving papers is shown here: level-1 papers (13%), level-2 papers (11%), level-3 papers (52%), and level-4 papers (24%); therefore, there were 24% prospective papers and 76% retrospective papers. Here, we present a part of the guideline on the five main surgical issues: indication to operation, operative procedure, peri-operative care, prognostic factor, and post-operative adjuvant therapy.

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Key words: Indication to operation; Hepatocellular carcinoma; Operative procedure; Peri-operative care; Prognostic factors

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INTRODUCTION

Surgeons may be severely criticized from the perspective of evidence-based medicine because the majority of surgical publications appear to not to be convincing[1]. In the top nine surgical journals in 1996, half of the 175 publications refer to pilot studies lacking a control group, 18% to animal experiments, and only 5% to randomized controlled trials (RCTs).

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Here, we present a part of the guideline on the five main surgical issues: indication to operation, operative procedure, peri-operative care, prognostic factor, and post-operative adjuvant therapy.

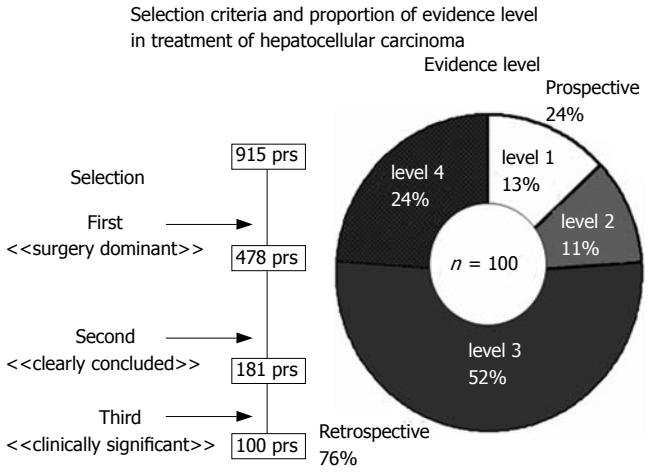


Figure 1 Selection criteria and proportion of evidence level in surgical treatment of hepatocellular carcinoma. We searched the literature using the Medline Dialog System with four keywords: HCC, surgery, English papers, in the last 20 years. The 915 publications were systematically reviewed by 3 steps. Almost all publications were retrospective (76%), there were only 24 (24%) with a high level evidence.

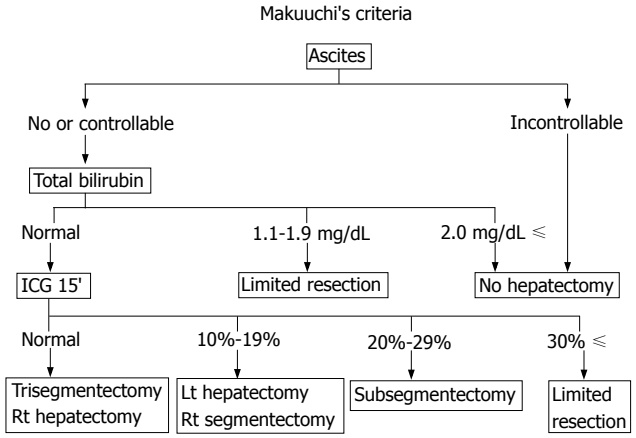


Figure 2 Algorithm before proceeding to safety hepatectomy for HCC with cirrhotic liver. Makuuchi's criteria include three factors: ascites, total serum bilirubin, and the ICG-R15: indocyanine green 15 min retention rate. This algorithm shows the maximal area for which an operation can be performed safely.

GUIDELINE 1: OPERATIVE INDICATION FOR HCC

Selection criteria in primary HCC

The selection of appropriate candidates for hepatectomy depends on careful assessment of the tumor status and liver function reserve. In order to evaluate tumor status, careful assessment of the liver function reserve is critical in patient selection for hepatectomy to avoid post-operative morbidity and mortality[3,4]. As selection criteria for operative procedures in patients with HCC and liver cirrhosis, Makuuchi's criteria have been used widely in Japan[5] (Figure 2). Criteria comprise ascites, total serum bilirubin, and the indocyanine green (ICG) K disappearance rate. In patients without ascites and with total bilirubin of < 2 mg, hepatectomy is indicated. The ICG rate indicates the upper limit of the hepatectomy procedure.

Table 1 The outcome of hepatectomy in major institutions

Author	Criteria	Morbidity (%)	Mortality (%)	5-yr Survival (%)
Blumgart[22]	Child	45	4.5	39
Bismuth H[7]	Child	40	10.0	48
Belghiti[8]	Child	31	6.4	37
Gozzetti[9]	Child	36	3.5	43
Bruix[10]	Child	-	3.9	51
Fan[6]	ICG15	38	2.5	49
Wu CC[11]	ICG15	11	2.3	51
Makuuchi[7]	ICG15	26	0.0	53

The Child classification is usually used by Western surgeons, while Eastern surgeons rely on the ICG-R15 retention rate to determine the liver function of patients. Operative morbidity rates were similar, but operative mortality rates were lower in the East, and five-year survival rates were higher in the East.

The clinical significance of the ICG clearance test was reported by Lau *et al*[6]. A prospective study was performed to identify the best test for assessing the adequacy of hepatic functional reserve in patients with HCC before hepatectomy. Each patient was evaluated before operation with regard to the ICG clearance test, the aminopyrine breath test, and the amino acid clearance test. ICG retention at 15 min was significantly associated to whether the patient survived or died.

As shown in Table 1, Eastern surgeons use the Child class and ICG test, while some Western centres rely on liver biochemistry and Child's classification alone to determine the liver function of patients. Operative morbidity rates were similar, but operative mortality rates were lower in the East, and five-year survival rates were higher in the East[7-11].

In fact, by refining pre-operative and post-operative care and surgical skills, liver resection mortality can be reduced to zero. There were 1056 hepatectomies without operative mortality and less than 3% major postoperative complications in HCC in the last eight years[12]. Liver resection can be performed without mortality provided that it is carried out in a high-volume centre and the surgeon pays sufficient attention to the liver functional reserve and the volume of liver to be removed. Thus, Makuuchi's criteria and the ICG clearance test are simple and reliable.

Early HCC

Early HCC is a well-differentiated cancer with no substantial destruction of the preexisting hepatic framework[13]. We performed a prospective study of surgical treatment for stage I HCCs to assess the therapeutic impact of hepatectomy. Stage I HCC was divided into two groups: microscopically early HCCs and others. The rate of microscopic regional spread, the time to recurrence, local recurrence, overall survival, and recurrence-free survival is significantly higher in early HCCs. This study shows that early HCC is a clinical entity with a high rate of surgical cure[14].

Portal vein tumor thrombus

The presence of portal vein tumor thrombus in a patient with hepatocellular carcinoma is one of the most significant factors for a poor prognosis[15-18]. In patients with portal vein tumor thrombus (PVTT), the median

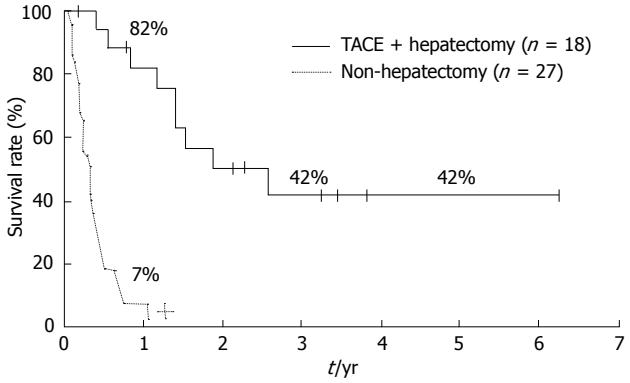


Figure 3 Treatment for HCC with portal vein tumor thrombus. Patient survival can be improved by TACE plus hepatectomy, when the number of primary nodules is not greater than two, the portal trunk is not occluded by tumor thrombus, and the indocyanine green retention rate at 15 min is > 20%. Minagawa *et al*. Ann Surg. 2001.

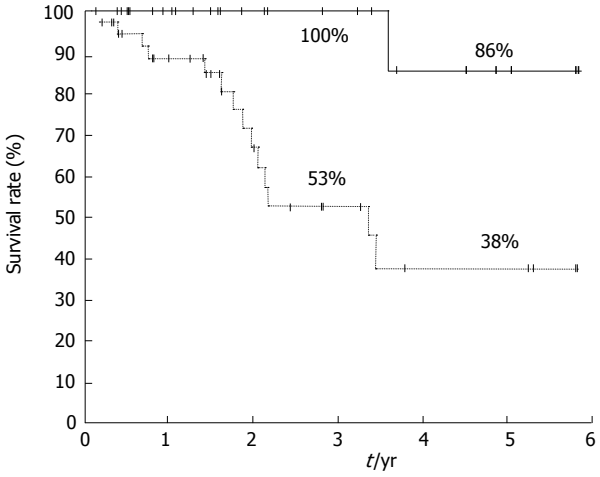


Figure 4 The result of repeat hepatic resection. Repeat hepatic resection is a satisfactory result in patients previously having undergone resection of a single HCC at primary resection and in whom recurrence developed after a disease-free interval of one year or more and the recurrent tumor had no portal invasion. Minagawa *et al*. Ann Surg. 2003.

survival was reported to be 2.7 mo, whereas survival in those without PVTT was 24.4 mo. Minagawa *et al*[19] reported that hepatic resection followed by pre-operative transarterial chemoembolization (TACE) leads to long-term survival in selected patients (Figure 3). The purpose of pre-operative transcatheter arterial embolization for patients with growth of PVTT is to interrupt the rapid growth of PVTT. They suggest that patient survival can be improved by this combination therapy, when the number of primary nodules is not higher than two, the portal trunk is not occluded by tumor thrombus, and the indocyanine green retention rate at 15 min is > 20%.

Repeat hepatic resection

The cumulative five-year recurrence rates after curative resection for HCC range from 77% to 100% according to the hepatitis virus and the main cause of late death. Some studies have demonstrated that repeat hepatectomy is effective for treating intrahepatic recurrent HCC in selected patients[20,21]. Using multivariate analysis, the authors showed that repeat hepatic resection is the treatment of choice for patients who have previously undergone resection of a single HCC at primary resection and in whom recurrence developed after a disease-free interval of one year or more and the recurrent tumor had no portal invasion[22] (Figure 4). For these reasons, repeat resection has been selected as the primary treatment for recurrent HCC under the same indications as for the first hepatectomy.

Transplantation

Liver transplantation is the best option for small HCC in patients presenting with severe deterioration of liver function. Currently, liver transplantation is considered to be the standard treatment for small HCC in patients with cirrhosis or advanced liver disease[23,24]. Optimal candidates for liver transplantation were identified in the '90 s: patients with single HCC of < 5 cm or with three or less nodules of 3 cm, without extrahepatic or vascular spread[25]. Liver transplantation eliminates or decreases significantly the risk of developing HCC, and it can prolong the life expectancy of the patient. In Japan, the scarcity of donor organs and the waiting time is too long, but living donor-related liver transplantation is rapidly becoming accepted. Moreover, cost-effectiveness analyses have shown that the benefits of living donor liver transplantation are achieved when the waiting time exceeds seven months.

Liver transplantation for HCC of < 5 cm in diameter has been shown to produce favorable survival results, but to extend the patient selection criteria for HCC, further evidence is required.

GUIDELINE 2: OPERATIVE PROCEDURE FOR HCC

Inflow vascular occlusion technique

Minimization of intraoperative blood loss is a widely accepted goal since the amount of blood loss and blood transfusion have been shown to adversely affect patient outcome[26,27]. Inflow occlusion techniques such as Pringle's maneuver and the hemihepatic vascular occlusion technique have been reported to decrease bleeding during transection of the liver parenchyma without causing damage to the remaining liver, and are widely accepted in conventional hepatectomy[28].

Makuuchi *et al* reported that by using the hemihepatic vascular occlusion technique, the amount of blood loss was markedly decreased[29]. The mean blood loss fell below 1000 mL, and the proportion of non-transfused patients reached 86.9% by 1986. This technique was followed by a prospective study[30] and RCT[31]. Man *et al* evaluated whether vascular inflow occlusion by the Pringle maneuver during hepatectomy is safe and effective in reducing blood loss by RCT[32]. The findings show a significant effect in less blood loss and better preservation of liver function during liver transection.

Anatomical resection

In 1985, Makuuchi devised "ultrasonically-guided

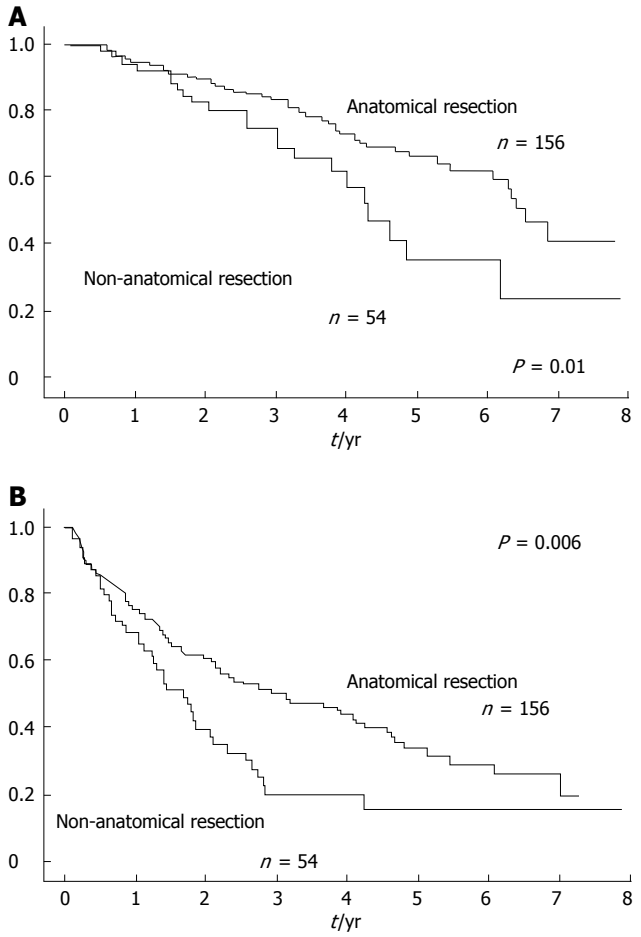


Figure 5 Anatomical resection in HCC. Overall (**A**) and disease-free (**B**) survival in anatomical and non-anatomical resection for single HCC. Both the five-year overall survival and disease-free survival rates in the anatomic resection group were significantly better than those in the non-anatomic resection group. Hasegawa *et al*. Ann Surg. 2003.

systematic segmentectomy," by which every Couinaud's segment could be removed completely[33]. This technique has been widely used around the world and is an essential technique in hepatic resection[34-40].

Anatomical resection is theoretically effective for eradicating the intrahepatic metastases of HCC with preference for eradicating portal venous tumor extension and intrahepatic metastases. This method is thought to have a beneficial effect on recurrence-free survival after hepatectomy for HCC. In the tumor enucleation group, the five-year survival rate was 35%, and in the segmentectomy group the five-year survival rate was 66% (group difference was significant). The relative risk of death in the segmentectomy group was 0.51, and segmentectomy appears thus to be the better option. The findings are supported by other retrospective studies[39-41].

Recently, Makuuchi's group showed that anatomic resection is a reasonable treatment option for HCC[42]. Both the five-year overall survival and disease-free survival rates in the anatomic resection group were significantly better than those in the non-anatomic resection group (Figure 5). Anatomical resection appeared to have a beneficial effect on recurrence-free survival after hepatectomy for HCC. Thus, systematic anatomical resection is the reasonable option of choice.

GUIDELINE 3: PERI-OPERATIVE CARE FOR HCC

Peri-operative nutritional support

Fan *et al* assessed by RCT whether peri-operative nutritional support improved the outcome in patients undergoing major hepatectomy for HCC associated with cirrhosis[43]. There was a reduction in the overall post-operative morbidity rate in the peri-operative nutrition group as compared with the control group. The operative morbidity rate was significantly lower in the supporting than in the control group (34% *vs* 55%).

Hemihepatic occlusion technique

Intermittent occlusion of hepatic blood inflow by means of the hemihepatic or total hepatic occlusion technique is essential for reducing operative blood loss, especially in cirrhotic patients[26-32]. Wu *et al* performed RCT to compare total versus hemihepatic vascular occlusion[44] (Table 2). There was no difference between the amount of blood loss during liver parenchymal transaction between total and hemihepatic vascular occlusion. However, the blood loss in the declamping period was significantly smaller in the hemihepatic vascular occlusion group due to the difference in operative blood loss and the incidence of blood transfusion. These findings indicate that hemi-hepatic clamping can be recommended for complex central liver resections in cirrhotic patients when the area of transaction is wide.

Blood transfusion

There are frequent recurrences of HCC after resection, especially in patients with cirrhosis. Dr. Yamamoto assessed retrospectively the effect of blood transfusion on HCC recurrence[45] (Figure 6). Recurrence-free survival was significantly better in the non-transfused than in the transfused group. Multivariate analysis showed that pre-operative blood transfusion was a significant predictor for accelerated recurrence of HCC after hepatectomy ($P = 0.003$). Thus, blood transfusion is avoided as far as possible[46,47].

Portal vein embolization

Portal vein embolization (PVE) before hepatectomy is intended to induce atrophy of the embolized lobe to be resected, with a compensatory hypertrophy of the counterlobe to be preserved[48]. To assess whether PVE is clinically useful, we determined the clinical outcome with PVE in 51 HCC patients[49]. After right lobe PVE, the mean ICG-R15 values rose by 4.3%, and the mean increase of the left lobe was 8%. All patients tolerated PVE well, and hepatic functional data returned to the baseline levels within one week. As a whole, the operative morbidity and mortality rates were the same as in the non-PVE hepatectomy group. The cumulative five-year survival rates were 44% in patients with HCC. PVE is a useful procedure for selected patients who may need extensive hepatectomy for diseased liver.

Table 2 The effect of hemihepatic occlusion in hepatectomy with cirrhotic patients (mean ± SE)

	Total inflow occlusion group (n = 28)	Hemihepatic occlusion group (n = 30)	P value
Resected liver weight (g)	443.9 ± 10.7 (150-1670)	422.7 ± 17.4 (180-1320)	0.29
Overall ischemic time (min)	96.0 ±10.9 (62-196)	94.2 ±9.9 (64.5-188)	0.28
Liver transection time (min)	86.4 ± 5.3 (64-171)	84.4 ± 5.2 (62-174.5)	0.54
Area of liver cut surface (cm^2)	103.1 ± 8.7 (62-208)	101.2 ± 6.9 (64-204)	0.86
Overall operative blood loss (mL)	6.82 ± 0.32 (3.8-11.5)	6.65 ± 0.26 (3.9-10.8)	0.37
Blood loss during parenchymal transection (mL)	1685 ± 170 (500-4800)	1159 ± 221 (400-4200)	0.049
Blood loss in declamping period (mL)	1015 ± 112 (400-4300)	974 ± 101 (300-4200)	0.51
Need for blood transfusion (n)	379 ± 12 (50-890)	179 ± 18 (50-460)	0.03
	12	5	0.04

Hemihepatic clamping is recommended for complex central liver resections when the area of transaction is wide. Wu *et al*. Arch Surg. 2002.

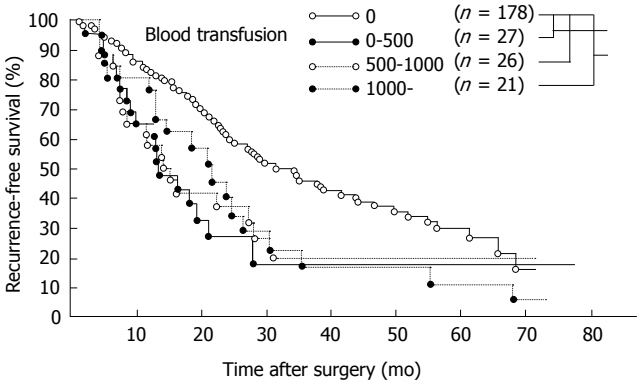


Figure 6 The relation between blood transfusion and recurrence. A close relationship between blood transfusion and recurrence-free survival was shown. Yamamoto *et al* . surgery 1994.

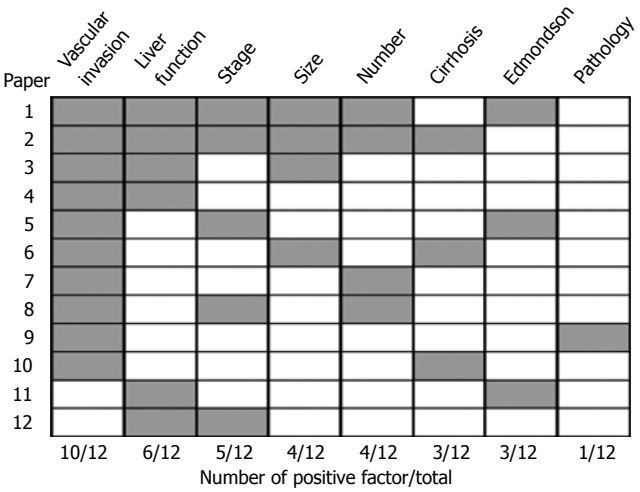


Figure 7 Prognostic factors in HCC. The eight main prognostic factors (vascular invasion, liver function, the stage of TNM classification, tumor diameter, number of tumor, degree of cirrhosis, Edmondson classification, and pathological type) were identified in twelve major publications. Vascular invasion, pTNM stage, and liver function are the most powerful prognostic factors.

GUIDELINE 4: PROGNOSTIC FACTORS IN HCC

There are many reports on prognostic factors in HCC after hepatectomy. We found 12 publications that focused on prognostic factors in HCC. Shown in Figure 7 are the eight prognostic factors identified: vascular invasion, liver function, pTNM stage, tumor size, number of tumor, degree of cirrhosis, Edmondson's classification, and pathological findings. Vascular invasion was a significant factor in multivariate analysis in ten of twelve publications. Liver function was significant in six publications, tumor stage in five, tumor size and number in four.

Vascular invasion has a high probability in recurrence-free survival, while tumor size is a controversial factor. Three factors, namely vascular invasion, pTNM stage, and liver function are the most powerful prognostic factors.

In a single HCC of < 2 cm in diameter, pathological early HCC showed a lower relative risk of recurrence (0.31) and death (0.26).

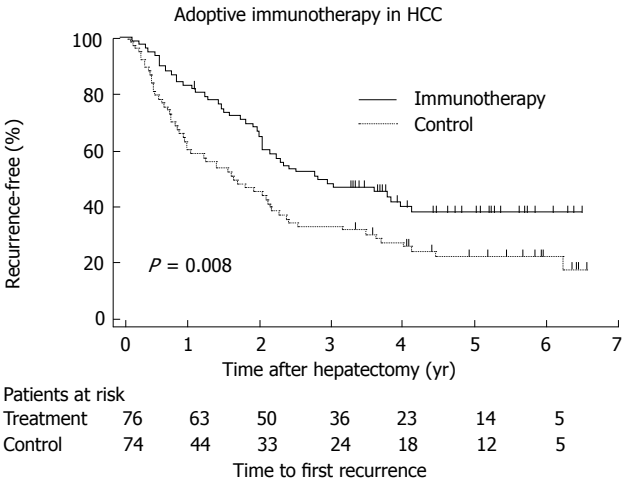


Figure 8 Adoptive immunotherapy in HCC. Autologous lymphocytes activated in vitro with recombinant interleukin-2 and infused five times during the first month. Adoptive immunotherapy reduces recurrence and improves recurrence-free survival after surgery for HCC. Takayama *et al*. Lancet 2000.

GUIDELINE 5: POSTOPERATIVE ADJUVANT THERAPY IN HCC

Resection of hepatocellular carcinoma is potentially curative, but local recurrence is common. In order to reduce the recurrence rate of HCC, effective adjutant therapy is needed. We found ten publications on RCT in adjuvant therapy[50-59]. Interventions included transarterial chemoembolization, retinoid therapy, immunotherapy (Figure 8), and interferon treatment[50-52]. However,

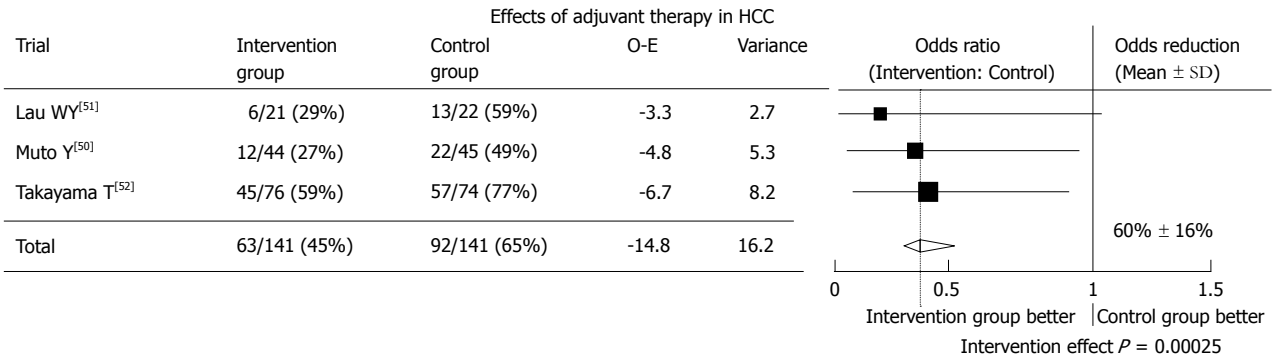


Figure 9 Effects of adjuvant therapy in HCC. Relative risk of recurrence in three RCTs by meta-analysis. The boxes show the relative risk, and the lines indicate 95% CI. A significant relative risk reduction was seen for recurrence (60%, SD 16%). O-E: observed-expected difference by Peto's method. Takayama *et al*. Cancer Reviews 2003.

the sample size was calculated only in three reports[60] (Figure 9). Eight reports showed positive results, but the quality of these RCTs are different.

Retinoid therapy

Muto *et al* reported that the oral intake of retinoid improves recurrence-free survival[50]. Cox proportional-hazards analysis demonstrated that, as an independent factor, polyprenoic acid reduced recurrence (relative risk, 0.31; 95% confidence interval, 0.12 to 0.78). Treatment with polyprenoic acid significantly reduced the incidence of recurrence after hepatectomy of the primary HCC as did a percutaneous injection of ethanol.

Intra-arterial ^{131}I-lipiodol injection

Lau *et al* reported a prospective randomized trial in which they aimed to determine whether one dose of postoperative adjuvant intra-arterial iodine-131-labeled lipiodol reduced the rate of local recurrence and increased disease-free and overall survival[51]. Patients who underwent curative resection for HCC and recovered within six weeks were given curative resection intra-arterial ^{131}I-lipiodol treatment. The median disease-free survival in the treatment and control groups was 57.2 (0.4-69.7) and 13.6 (2.1-68.3) mo, respectively (P = 0.037). Three-year overall survival in the treatment and control groups was 86.4% and 46.3%, respectively (P = 0.039). ^{131}I-lipiodol had no significant toxic effect, and this treatment decreases the rate of recurrence and increases disease-free and overall survival.

Adoptive immunotherapy

We used adoptive immunotherapy. From an HCC patient, peripheral blood was taken and lymphocytes were cultured in the presence of interleukin-2 for two weeks before assay-activated autologous T-cells were transferred back to the same patient[52]. Before the start of this RCT, we calculated the sample size of 143 patients to detect 20% improvement of recurrence-free survival at 5% significance and 80% power. After a median follow-up of five years, adoptive immunotherapy improved recurrence-free survival (Figure 8). On multivariate analysis, the relative risk of recurrence was 0.60.

CONCLUSION

Operative procedures are selected by precise pre-operative evaluation of hepatic functional reserve; thus, we rely on the algorithm (Figure 2) for decision making in surgical treatment. The overall five-year survival after resection of HCC in a high-volume centre is around 50%. However, the disease-free survival after partial hepatectomy is unsatisfactory because of the high incidence of intrahepatic recurrence. As there is no established adjuvant therapy that is proven to be effective in prevention of recurrence, aggressive management of recurrence using various modalities including surgical resection, transarterial chemoembolization, and local ablation is currently the most practical measure in prolonging survival after resection of HCC.

Liver transplantation offers the dual advantages of eradicating the tumor and replacing the diseased liver. This modality has become the treatment of choice, especially for patients with end-stage liver disease, whereby LDLT is a promising treatment for HCC in Japan.

We here presented part of the Japanese guideline, showing that surgery is the primary option of choice for HCC. However, the guideline should be refined using high-level evidence as it becomes available.

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Effects of Wei Chang An on expression of multiple genes in human gastric cancer grafted onto nude mice

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Abstract

AIM: To investigate the expression of multiple genes in Chinese jianpi herbal recipe Wei Chang An (WCA) in human gastric cancer cell line SGC-7901.

METHODS: A human gastric adenocarcinoma cell line SGC-7901 grafted onto nude mice was used as the animal model. The mice were randomly divided into 3 groups, one control and the two representing experimental conditions. Animals in the two experimental groups received either WCA over a 34-d period or 5-fluorouracil (5-FU) over 6-d period starting at 8th d after grafting. Control animals received saline on an identical schedule. Animals were killed 41 d after being grafted. The expression profiles in paired WCA treated gastric cancer samples and the N.S. control samples were studied by using a cDNA array representing 14181 cDNA clusters. The alterations in gene expression levels were confirmed by Real-time Quantitative polymerase chain reaction (qPCR).

RESULTS: When compared with controls, the average tumor inhibitory rate in WCA group was 44.32% ± 5.67% and 5-FU 47.04% ± 11.33% ($P < 0.01$, respectively). The average labeling index (LI) for PCNA in WCA group and 5-FU group was significantly decreased compared with the control group. Apoptotic index (AI) was significantly increased to 9.72% ± 4.51% using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescence nick end labeling (TUNEL) method in WCA group compared with the controls 2.45% ± 1.37%. 5-FU group was also found to have a significantly increased AI compared with the controls. The expression of cleaved Caspase-3 in WCA group and 5-FU group was significantly increased compared with the control group respectively. There were 45 different expressed sequence tags (ESTs) among the control sample pool and WCA sample pool. There were 24 ESTs up-regulated in WCA samples and 21 ESTs down-regulated. By using qPCR, the expression level of Stat3, rap2 interacting protein x (RIPX), regulator of differentiation 1 (ROD1) and Bcl-2 was lower in WCA group than that in control group respectively. By using SP immunohistochemical method the expression of Phospho-Stat3 (Tyr705) and Bcl-2 in WCA group and 5-FU group was significantly decreased compared with the control group respectively.

CONCLUSION: WCA could inhibit gastric cancer cell SGC-7901 growth *in vivo*. WCA could induce gastric cancer cell apoptosis and suppress proliferation. Its mechanisms might be involved in the down-regulation of Stat3, RIPX, ROD1 and Bcl-2 gene.

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Key words: Gastric cancer; Chinese Jianpi herbs; Expressions of multiple genes; Real-time quantitative PCR

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INTRODUCTION

Gastric cancer, despite its declining incidence, remains one of the most common causes of cancer-related death worldwide. Overall, 5-year survival after the diagnosis of gastric cancer is 10%-21%[1]. Surgery is the main method of treatment. Patients who undergo a potentially curative resection have a better prognosis, but even with curative resection, gastric cancer still has a high risk of relapse.

Although various chemotherapeutic agents have been reported for use in patients with gastric cancer, the median prognosis for survival in patients with advanced gastric cancer remains less then 7-12 mo at present[2-5].

We developed a Chinese jianpi herbal recipe Wei Chang An (WCA) to improve survival of gastric cancer patients. Previous clinical paired comparative studies have indicated patients with gastric carcinoma will benefit from WCA treatment[6,7]. Clinical data have shown that WCA can increase the 3-year survival rate of advanced gastric cancer. Another clinical study of 208 patients with gastric adenocarcinoma has suggested interaction between TNM stage (P = 0.000), radical resection (P = 0.000), chemotherapy (P = 0.002) and WCA (P = 0.000) in their effect on long-term survival. Patients who have received WCA have better prognosis on multivariate analysis, independent of other prognostic factors. The odds ratios of WCA was 0.315 (95% CI 0.204-0.486)[8].

In an animal model of human gastric cancer cell line SGC-7901 subcutaneously grafted onto nude mice, we have found tumor growth is significantly inhibited by treatment with WCA. Immunohistochemical staining for Ki-67 has shown WCA inhibits cell proliferation. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescence nick end labeling (TUNEL) method, flow cytometry and electron microscopy have clarified that WCA also enhances apoptosis[9]. In the orthotopic implanted model, inhibition of tumor growth and metastasis has also been verified, and higher activity of NK cells has been found in WCA-treated animals compared with the control group[10-12]. Based on previous studies, we inferred that the tumor suppressive effect of WCA involves multiple biological processes and mechanisms. This study was designed to investigate the underlying mechanism of WCA in treatment of gastric cancer, using the cDNA array technique, real-time quantitative PCR and immunohistochemical techniques.

MATERIALS AND METHODS

Drugs

The composition of WCA includes *Atractylodes macrocephala* koidz., *Poria cocos* (Schw.) Wolf, *Glycyrrhiza uralensis* Fisch., Rehd. Et Wils., and *Prunella vulgaris* L. The preparation of WCA decoction has been described previously[8,9] and the concentration of the decoction was 120 g/L. High Performance Liquid Chromatography (HPLC) was used for monitoring the stability of the decoction. Lichrospher-C18 column at 25℃ was used. The mobile phase consisted of methyl alcohol/water with a linear gradient as follows: methyl alcohol, 5, 5, 70, 100, 100%; water, 95, 95, 30, 0, 0%; at 0, 5, 15, 35 and 40 min, respectively, and the flow rate was 1 mL/min. The detection was performed at UV 280 nm (Figure 1). 5-fluorouracil (5-FU) was purchased from Xudong-Haipu (Shanghai) Pharma, China (Lot 030601).

Cell line, animal model and experimental schedule

A human gastric adenocarcinoma cell line SGC-7901 and 73 6-7-wk-old male BALB/C-nu/nu mice (weight 18-22 g) were obtained from Shanghai Tumor Institute [No. SCXK (Shanghai) 2002-0001; Shanghai, China]. The animal experiment was repeated three times. The mice were divided into three groups in every test, one control and two experimental. Animals in the two experimental groups received either WCA 0.5 mL/d by gastric perfusion over a 34 d period or 5-FU 20 mg/kg per day i.p., over a 6-d period starting on day 8 after grafting. Control animals received saline 0.5 mL/d by gastric perfusion on an identical schedule. Animals were killed 41 d after being grafted. Tumor weight was determined immediately by electron balance after the animals were killed. Tumor tissue was obtained within 2 min after removal from the animal. Each block was cut into three pieces, one for routine pathological diagnosis and immunohistochemical staining, and the others for molecular analysis. The latter samples were frozen immediately in liquid nitrogen and stored at -260℃.

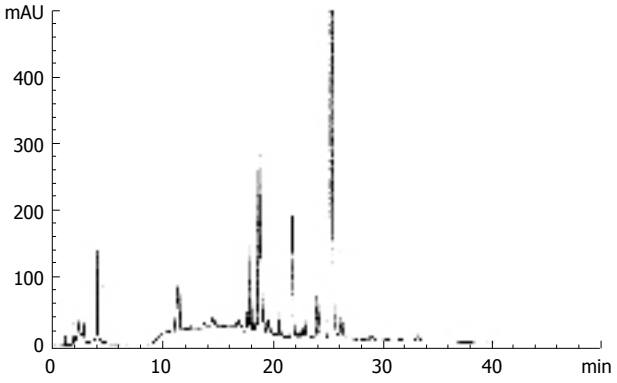


Figure 1 HPLC print of 120 mg/mL WCA. HPLC: High Performance Liquid Chromatography; WCA: Wei Chang An.

Cell proliferation and apoptosis

Cell proliferation: The streptavidin peroxidase (SP) immunohistochemical method was used to detect the expression of proliferating cell nuclear antigen (PCNA) in xenografts. The dilution of PCNA mouse monoclonal antibody (Shanghai Changdao Biotech, Shanghai, China; Cat. No. M-0437, Lot 0509) was 1:150. The procedure was performed according to the manufacturer's instructions. The positive cells were identified, counted and analyzed under the light microscope. Non-necrotic zones were selected in the tissue section. Five images (× 400) of at least 1000 cells were selected on the screen and analyzed. PCNA is predominantly localized in the nuclei.

Apoptosis: Apoptotic index (AI) was examined by TUNEL method (Roche Diagnostics, GmbH, Mannheim, Germany; Cat. No. 11 684 817 910, Lot 12 067 000). The procedure was carried out according to the kit protocol. The positive cells were identified, counted and analyzed under the light microscope. Non-necrotic zones were selected in the tissue sections. Five images of at least 1000 cells were selected on the screen and analyzed.

Expression of cleaved caspase-3: SP immunohisto-

Table 1 Xenograft tumor weight in nude mice after different treatments

Group	Test 1				Test 2				Test 3			
	Number Begin/end	Tumor weight (g)	*P* value	Inhibition rate (%)	Number Begin/End	Tumor weight (g)	*P* value	Inhibition rate (%)	Number Begin/End	Tumor weight (g)	*P* value	Inhibition rate (%)
WCA	8/8	0.99 ± 0.76	0.0117[a]	48.7	8/7[2]	1.02 ± 0.16	0.0045[b]	37.91	8/8	0.88 ± 0.47	0.0072[b]	46.35
5-FU	10/9[1]	0.77 ± 0.48	0.0002[b]	60.1	7/7	0.97 ± 0.34	0.0056[b]	41.29	7/7	0.98 ± 0.46	0.0167[a]	39.74
Control	8/8	1.93 ± 0.58			8/8	1.65 ± 0.46			9/9	1.63 ± 0.52		

Data are expressed in mean ± SD. [a]$P < 0.05$ *vs* control group; [b]$P < 0.01$ *vs* control group. [1]Died of toxicity of 5-FU; [2]Died of the operation of gastric perfusion.

chemistry was also used to detect cleaved caspase-3. The dilution of cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology, Beverly, MA, USA; Cat. No. 9661, Lot 15) was 1:200. Cleaved caspase-3 was predominantly localized in the cytoplasm and perinuclear region in apoptotic cells.

cDNA microarray

The expression profiles in paired WCA-treated gastric cancer and control samples were studied by using a cDNA array (Casarray, Shanghai, China) representing 14 181 cDNA clusters.

Membranes were exposed to a phosphor screen overnight and scanned using a FLA-3000A fluorescent image analyzer (Fuji Photo Film, Tokyo, Japan). After subtracting the background selected from an area in which no PCR product was spotted, clones with an intensity density over 10 were considered as positive signals. Hybridization data were considered invalid if there was a difference of over 1.5-fold in the intensity of any of the 12 control spots for the same control cDNA between arrays.

The hybridization intensity of corresponding dots in control and WCA groups was compared. If the difference in spot intensity in these two groups was more than twofold higher or lower, the corresponding genes were considered as being differentially expressed.

Real-time quantitative PCR

According to the result of cDNA array analysis, 19 genes related to regulation of cell proliferation, differentiation and apoptosis were selected for investigation. Quantification of gene expression levels were confirmed by real-time quantitative PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Sequences of the primers used for RT-PCR analysis are described in Table 1. Primers were designed by Primer Express 2.0 and synthesized by TaKaRa Biotechnology (Dalian), China. Total cellular RNA was isolated from tumor tissues using Trizol (Gibco/BRL, Carisbad, USA). The RNA extracted from each sample was qualified by agarose gel electrophoresis and ethidium bromide staining and the A: 260/280 ratio was determined by electrophotometry. The extracted RNA was converted to first strand cDNA with AMV reverse transcriptase (Reverse Transcription System, Promega, Madison, USA). Nineteen genes and GAPDH were amplified using a sequence detection system (ABI PRISM® 7900HT; ABI, Foster City, CA, USA). Each cDNA sample was placed in three different wells (triple-offset). The PCR reaction consisted of stage 1, 50℃ for 2 min; stage 2, 95℃ for 15 min; stage 3, 40 cycles of 94℃ for 15 s, 55℃ for 30 s, and 72℃ for 30 s; stage 4, 95℃ for 15 s, 60℃ for 15 s and 95℃ for 15 s. Each reaction tube contained: 10 μmol/L primer (a pair) 0.5 μL + SYBR 2.5 μL + ROX 0.1 μL + cDNA 1 μL + ddH_2O 0.9 μL (Platinum®SYBR® Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA, USA; Cat. No. 11733-038). Relative gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method[13].

Immunohistochemical assay

The SP immunohistochemical method was used to detect Phospho-Stat3 and Bcl-2 expression in tumor cells. The dilution of Phospho-Stat3 (Tyr705) antibody (Cell Signaling Technology; Cat. No. 9131, Lot 5) was 1:100 and Bcl-2 (Shanghai Changdao Biotech; Cat. No. M-0025, Lot 51806) was 1:50. Phospho-Stat3 was predominantly localized in the nucleus. Bcl-2 was predominantly localized in the cytoplasm and nuclear membrane.

Statistical analysis

Statistical analysis was performed using SPSS ver. 11.0 (SPSS, Chicago, IL, USA). The results were expressed as mean ± SD and significant difference was assessed by Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

WCA-induced effects on tumor growth

When compared with controls, tumor growth was significantly inhibited by treatment with WCA or 5-FU ($P < 0.01$). The average tumor inhibition rate in the WCA group was 44.32% + 5.67% and 47.04% ± 11.33% in the 5-FU group (Table 1).

Cell proliferation and apoptosis

The average labeling index for PCNA in the WCA and 5-FU groups was significantly decreased compared with that in the control group (Table 2, Figure 2). AI of human gastric cancer xenografts in nude mice was significantly increased to 9.72% ± 4.51% in the WCA group compared with that in the control group (2.45% ± 1.37%). The 5-FU group also had a significantly increased AI compared with the controls (Table 2, Figure 2). The expression of cleaved caspase-3

Table 2 WCA-induced effects on gastric cancer cell SGC-7901

Group	*n*	PCNA						TUNEL		Cleaved caspase-3 (Asp 175) (%)	
		Positive rate (%)	*P* value	Intense positive rate (%)	*P* value	Total positive rate (%)	*P* value	Apoptotic index (%)	*P* value	Positive rate (%)	*P* value
WCA	8	35.73 ± 6.01	0.0005[b]	3.39 ± 1.48	0.0155[a]	39.03 ± 7.37	0.0009[b]	9.72 ± 4.51	0.0007[b]	5.20 ± 2.26	0.0367[a]
5-FU	9	37.86 ± 16.50	0.0325[a]	5.62 ± 4.21	0.1972	43.48 ± 19.77	0.0406[a]	5.74 ± 1.75	0.0007[b]	4.73 ± 1.76	0.0451[a]
Control	8	53.48 ± 9.34		8.78 ± 5.43		62.26 ± 13.80		2.45 ± 1.37		2.82 ± 1.84	

Data are expressed in mean ± SD. [a]$P < 0.05$ *vs* control group; [b]$P < 0.01$ *vs* control group.

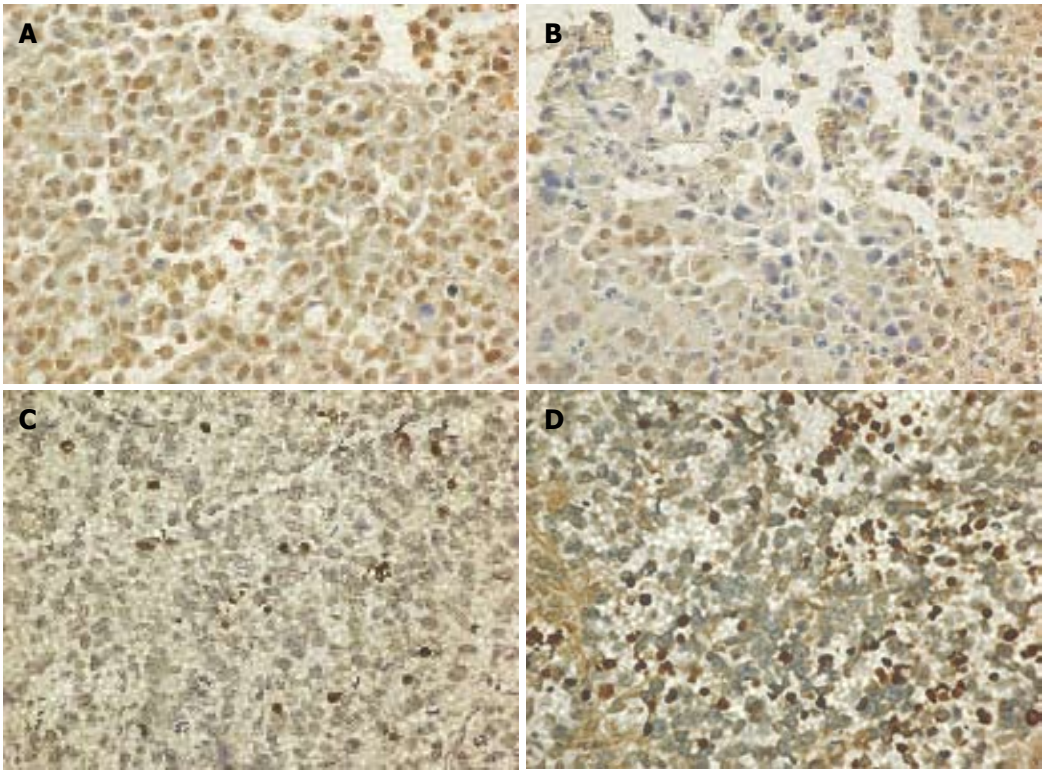


Figure 2 PCNA expression detected by IHC (**A**, **B**) and Apoptotic cell detected by TUNEL (**C**, **D**) in various groups of human gastric gastric adenocarcinoma cell line SGC-7901 grafted onto nude mouse. PCNA is predominantly localized at nuclei. **A**: NS Control group (IHC, × 400); **B**: WCA group (IHC, × 400); Apoptotic cell labeled in various groups; **C**: NS Control group (TUNEL, × 400); **D**: WCA group (TUNEL, × 400).

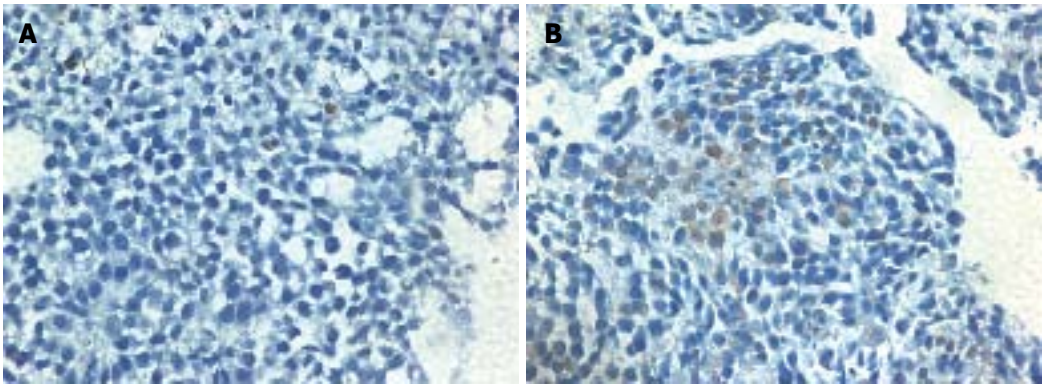


Figure 3 Cleaved Caspase-3 (Asp 175) expression in various groups of xenografts. Cleaved Caspase-3 is predominantly localized at cytoplasmic and perinuclear. **A**: NS Control group (IHC, × 400); **B**: WCA group (IHC, × 400).

in the WCA and 5-FU group was significantly increased compared with that in the control group (Table 2, Figure 3).

Confirmation of cDNA array findings

There were 45 different expressed sequence tags (ESTs) among the control and WCA samples. There were 24 ESTs up-regulated and 21 down-regulated in the WCA samples. These 45 ESTs contained 35 cloned genes and 11 unknown ESTs. According to the results of cDNA array analysis, 19 genes related to regulation of cell proliferation, differentiation and apoptosis were selected for investigation. Quantification of gene expression levels was confirmed by real-time quantitative PCR. The expression level of *stat3* ($2^{-\Delta\Delta CT} = 0.16$), rap 2 interacting protein x (RIPX) ($2^{-\Delta\Delta CT} = 0.18$), regulator of differentiation 1 (ROD1) ($2^{-\Delta\Delta CT} = 0.23$) and *bcl-2* ($2^{-\Delta\Delta CT} = 0.10$) was

Table 3 Quantification of gene expression by real-time quantitative PCR assay

Gene name	UniGene cluster	Primer sequences (5′-3′)		Combining sites (bp)	Amplifiers (bp)	WCA (120 mg/mL) *vs* NS control	
						ΔΔCT (*n*)	$2^{-\Delta\Delta CT}$
stat3	Hs.421342	forward	CCTGGAGCAGCTCCATCAG	254	58	2.65 (4)	0.16 (0.11-0.24)
		reverse	AAACTGCCGCAGCTCCATT	311			
RIPX	Hs.7927	forward	GAGTGCCTTTAAGCTGCAGAGTT	6	69	2.50 (5)	0.18 (0.13-0.23)
		reverse	TCCAAGCGACTGTTTAGTTCACTT	74			
ROD1	Hs.269988	forward	AACTCCTCTCTGTAAAGCATTTTGC	525	64	2.09 (4)	0.23 (0.12-0.23)
		reverse	TGCACTGGGTCTTCTTTCAGAA	588			
bcl-2	Hs.12677	forward	TGTTGGCCGGATCACCAT	2557	60	3.27 (5)	0.10 (0.06-0.17)
		reverse	TCCCCAATGATCAGGTCCTTT	2616			

Table 4 Xenograft gastric cancer cell SGC-7901 P-Stat3 and *Bcl-2* expression after different treatments

Group	*n*	P-Stat3						Bcl-2	
		Positive rate (%)	*P* value	Intense positive rate (%)	*P* value	Total positive rate (%)	*P* value	Positive rate (%)	*P* value
WCA	8	35.93 ± 12.67	0.0024[b]	3.64 ± 1.72	0.0023[b]	39.57 ± 13.31	0.0002[b]	1.62 ± 0.82	0.0006[b]
5-FU	9	36.95 ± 27.21	0.0732	4.38 ± 3.62	0.0050[b]	41.33 ± 30.22	0.0243[a]	7.72 ± 5.31	0.9364
Control	8	56.49 ± 9.34		13.16 ± 7.06		69.65 ± 10.80		7.53 ± 3.73	

Data are expressed in mean ± SD. [a]$P < 0.05$ *vs* control group; [b]$P < 0.01$ *vs* control group.

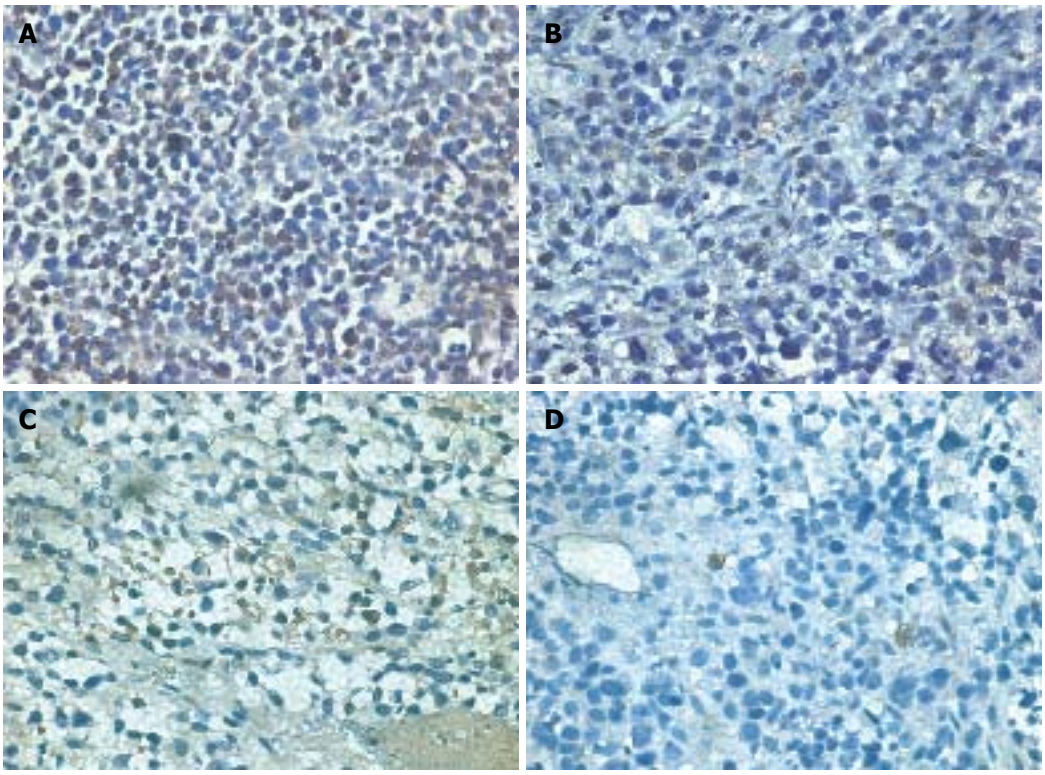


Figure 4 Phospho-Tyr705-Stat3 and Bcl-2 expression in various groups of human gastric adenocarcinoma cell line SGC-7901 grafted onto nude mouse detected by IHC. P-Stat3 is predominantly localized at nuclei. Bcl-2 is predominantly localized at cytoplasmic and nuclear membrane. **A**: NS Control group: positive staining of P-Stat3 in xenograft cells (IHC, × 400); **B**: WCA group: positive staining of P-Stat3 in xenograft cells (IHC, × 400); **C**: NS Control group: positive staining of Bcl-2 in xenograft cells (IHC, × 400); **D**: WCA group: positive staining of Bcl-2 in xenograft cells (IHC, × 400).

lower in the WCA group than that in the control group. The expression level of another 15 genes did not differ between the two groups (Table 3).

Expression of Phospho-Stat3 (Tyr705) and Bcl-2

We performed immunohistochemistry to confirm our data at the protein level. Expression of Phospho-Stat3 (Tyr705) and Bcl-2 was significantly decreased in the WCA and 5-FU group compared with that in the control group (Table 4, Figure 4).

DISCUSSION

Gastric cancer is the second most common cancer worldwide. Its poor outcome is due to the fact that 75% of patients are considered incurable at diagnosis (advanced

disease), therefore, its treatment remains a great challenge. The Chinese jianpi herbal recipe WCA has been developed to improve survival of gastric cancer patients. Previous studies have shown the tumor suppressive effect of WCA involved multiple biological processes and mechanisms, including enhanced apoptosis and inhibition of proliferation. However, further mechanisms are not fully understood[9-12].

In the present study, tumor growth was significantly inhibited by treatment with WCA at a concentration of 120 g/L in the animal model of human gastric cancer cell line SGC-7901 grafted onto nude mice ($P < 0.01$). The average tumor inhibitory rate in the WCA group was 44.32% ± 5.67%. The TUNEL method was used to detect apoptotic cells. AI of cancer cells was significantly increased in the WCA group compared with that in the control group. The 5-FU group also showed a significantly increased AI compared with that in the control group. Caspase-3 is one of the key executioners of apoptosis, which is either partially or totally responsible for proteolytic cleavage of many key proteins, such as the nuclear enzyme poly ADP-ribose polymerase. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 subunits. Cleavage of caspase-3 requires aspartic acid at the P1 position[14,15]. Cleaved caspase-3 (Asp175) antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3, which results from cleavage adjacent to Asp175. It does not recognize full-length caspase-3 or other cleaved caspases. The expression of cleaved caspase-3 in the WCA and 5-FU groups was significantly increased compared with that in the control group. This result clarifies that WCA enhances apoptosis.

Immunohistochemical staining for PCNA also showed WCA inhibited cell proliferation. These results were consistent with our previous studies[9].

Carcinogenesis and the progression of carcinoma are thought to develop from multistage activation of oncogenes and loss of suppressor genes. In trying to understand the mechanism of WCA at the RNA expression level, we used cDNA microarray and real-time quantitative PCR techniques. The expression profiles in paired WCA-treated gastric cancer and control samples were screened by cDNA array analysis, which represented 14181 cDNA clusters, and verified by real-time quantitative PCR and immunohistochemistry. There were 24 ESTs up-regulated and 21 ESTs down-regulated in WCA samples. These 45 ESTs contains 35 cloned genes and 11 unknown ESTs. According to the result of cDNA microarray analysis, 19 genes related to the regulation of cell proliferation, differentiation and apoptosis were selected for investigation. The expression level of Stat3 ($2^{-\Delta\Delta CT} = 0.16$), RIPX ($2^{-\Delta\Delta CT} = 0.18$), ROD1 ($2^{-\Delta\Delta CT} = 0.23$) and Bcl-2 ($2^{-\Delta\Delta CT} = 0.10$) was lower in the WCA group than that in the control group. The expression level of another 15 genes did not differ between these two groups. Moreover, we performed immunohistochemistry to confirm these data at the protein level. The expression of Phospho-Stat3 (Tyr705) and Bcl-2 in the WCA and 5-FU groups was significantly decreased compared with that in the control group.

Jak-Stat pathways play important roles in oncogenesis, tumor progression, angiogenesis, cell motility, immune responses and stem cell differentiation[16]. Stat3 is a key signaling molecule for many cytokines and growth-factor receptors, and is required for murine fetal development. In addition, Stat3 is constitutively activated in a number of human tumors and possesses oncogenic potential and anti-apoptotic activity[17,18]. Stat3 is activated by tyrosine phosphorylation at Tyr705, which induces dimerization, nuclear translation and DNA binding. Stat3 isoform expression appears to reflect biological function. The relative expression levels of Stat3 α (86 kDa) and Stat3 β (79 kDa) depends on cell type, ligand exposure or maturation stage of the cells. Phospho-Stat3 (Tyr705) antibody detects endogenous levels of Stat3 only when phosphorylated at Tyr705. Activated forms of Stat3 and Phospho-Stat3 (Tyr705) were found in the nucleus of cancer cells in 30% of human gastric cancer specimens, which suggests that constitutively activated Stat3 signaling supports gastric cancer cell survival in association with survivin expression[19]. Stat3 regulates multiple genes important for apoptosis, cellular proliferation and angiogenesis in gastric cancer, therefore its activation may play an important role in gastric cancer development and progression[20]. The expression level of *stat3* mRNA and Phospho-Stat3 protein was lower in the WCA group than that in the control group, which suggests WCA may regulate human gastric cancer cell SGC-7901 apoptosis and/or proliferation through the Stat3 pathway.

The expression level of *bcl-2* mRNA was also decreased in the WCA group, as was that of its encoded protein. Bcl-2 is one of the Bcl-2 family that is important in regulation of apoptosis. Previous studies have demonstrated Stat3 regulates Bcl-2 expresssion[21], therefore, we inferred that the decrease in Bcl-2 expression in the WCA group was related to the mechanism of action of WCA, and may be correlated with lower Stat3 expression.

Other two genes: ROD1 (Hs.269988) or RIPX (Hs.7927) we found decreased expressing in WCA group had been seldom studied in solid tumor. In the fission yeast *Schizosaccharomyces pombe*, the *N*-arginine dibasic convertase 1 (NRD1) gene that encodes an RNA-binding protein negatively regulates the onset of differentiation. The mammalian homologue of NRD1 is ROD1, which encodes a protein with four repeats of typical RNA-binding domains. When expressed in fission yeast, ROD1 protein functions are similar to those of Nrd1. ROD1 is highly expressed in adult and embryo hematopoietic cells or organs. Overexpression of ROD1 effectively blocks the differentiation of human leukemia cells, without affecting their proliferative ability, which suggests ROD1 plays a critical role in controlling differentiation in mammalian cells[22,23]. Andoh *et al* have reported interaction of Rod1 and Rsp5 may be important for drug resistance[24]. However, the role of ROD1 in the development of human gastric cancer is not clear.

Another name for RIPX is kIAA0871, which is a member of the KIAA gene family[25]. In the progress of

the human gene project, KIAA genes have been found continuously. These genes are newly identified long cDNAs that encode large proteins. The large proteins play important roles in biological processes. Further physiological, developmental and genetic studies are necessary[26].

In conclusion, genes involved in the mechanism of WCA are Stat3, Bcl-2, RIPX and ROD1. WCA down-regulates the expression of several genes. How WCA affects the expression of these genes and the roles of these genes themselves need to be explored.

COMMENTS

Background

Previous clinical paired comparative studies have indicated patients with gastric carcinoma will benefit from treatment with the Chinese Jianpi herbal recipe Wei Chang An (WCA).

Research frontiers

This study was designed to investigate the underlying mechanism of WCA in treatment of gastric cancer by cDNA microarray analysis and real-time quantitative polymerase chain reaction.

Applications

Gastric cancer, despite its declining incidence, remains one of the most common causes of cancer-related death throughout the world. The poor outcome is due to the fact that 75% of patients are considered incurable at diagnosis (advanced disease), and therefore, its treatment remains a great challenge. Previous research has shown the tumor suppressive effect of WCA involves multiple biological processes and mechanisms, including enhanced apoptosis and inhibition of proliferation. To understand the further mechanisms of WCA is of benefit to developing a new therapeutic method.

Terminology

WCA is composed of *Atractylodes macrocephala* koidz., *Poria cocos* (Schw.) Wolf, *Sargentodoxa cuneata* (Oliv.) Rehd. Et Wils. and *Prunella vulgaris* L. The concentration of the decoction was 120 g/L. High Performance Liquid Chromatography was used for monitoring the stability of WCA.

Peer review

This is an interesting study. The authors investigated the expression of multiple genes in nude mice grafted with human gastric cancer cell line SGC-7901 and treated with WCA. It was demonstrated that WCA inhibited gastric cancer cell growth *in vivo*. WCA can induce gastric cancer cell apoptosis and suppress proliferation.

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Influence of quasispecies on virological responses and disease severity in patients with chronic hepatitis C

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Abstract

AIM: To elucidate the influence of quasispecies on virological response and disease severity in patients with chronic hepatitis C.

METHODS: Forty seven patients with hepatitis C [32 with chronic active hepatitis (CAH), 9 with cirrhosis, and 6 with hepatocellular carcinoma (HCC)] were screened for the presence of quasispecies by single stranded conformational polymorphism (SSCP) analysis in the hypervariable region (HVR) and non-structural 5B (NS5B) viral genes of hepatitis C virus. The 41 patients excluding those with HCC were on therapy and followed up for a year with the determination of virological response and disease severity. Virus isolated from twenty three randomly selected patients (11 non-responders and 12 showing a sustained virological response) was sequenced for the assessment of mutations.

RESULTS: The occurrence of quasispecies was proportionately higher in patients with HCC and cirrhosis than in those with CAH, revealing a significant correlation between the molecular evolution of quasispecies and the severity of disease in patients with hepatitis C. The occurrence of complex quasispecies has a significant association ($P < 0.05$) with the non-responders, and leads to persistence of infection. Significant differences ($P < 0.05$) in viral load (log_{10} IU/mL) were observed among patients infected with complex quasispecies (CQS), those infected with simple quasispecies (SQS) and those with no quasispecies (NQS), after 12 wk (CQS-5.2 ± 2.3, SQS-3.2 ± 1.9, NQS-2.8 ± 2.4) and 24 wk (CQS-3.9 ± 2.2, SQS-3.0 ± 2.2, NQS-2.1 ± 2.3) in the HVR region. However, a statistically significant difference ($P < 0.05$) was observed between the viral loads of patients infected with CQS and those infected with NQS in NS5B viral gene after 24 wk (CQS-3.9 ± 2.2, SQS-3.0 ± 2.2, and NQS-2.1 ± 2.3) and 48 wk (CQS-3.1 ± 2.7, SQS-2.3 ± 2.4, NQS-2.0 ± 2.3) of therapy. Disease severity was significantly associated with viral load during therapy. The strains isolated from non-responders showed close pairing on phylogeny based on the NS5B gene, but dissimilar HVR regions. This revealed the possibility of the selection of resistant strains during the evolution of quasispecies in NS5B.

CONCLUSION: Viral quasispecies may be an important predictor of virological responses to combination therapy in patients with chronic hepatitis C. Complex quasispecies and resistant strains may lead to high viral loads during therapy, with a concerted effect on disease severity.

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Key words: Hepatitis C virus; Single-strand conformational polymorphism; Quasispecies; Virological response; Interferon therapy

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INTRODUCTION

Hepatitis C is a noncytopathic hepatotrophic virus having a single stranded, positive sense linear -9.5 kb RNA genome. Hepatitis C virus infection is the leading cause of acute, chronic, liver cirrhosis and hepatocellular carcinoma. Chronic infection due to hepatitis C virus (HCV) occurs in 170-200 million people worldwide[1,2]. In India, 15%-20% of all patients with chronic liver disease and 5%-10% of hepatocellular carcinoma patients are infected with hepatitis C[3]. The HCV continues to persist, unless the patients are put on interferon therapy. Treatment with interferon-based

therapy and error-prone replication of the genome *via* RNA-dependent RNA polymerase (RDRP) accelerates the evolution of HCV variants during the course of infection[2]. The origination of highly heterogeneous population in hepatitis C infected patients closely related to HCV genome because of interferon therapy, immune pressure and replication strategy called quasispecies[4].

The hypervariable region 1 (HVR-1) is located at the 5'-end of the E2 envelope glycoprotein, and variations occur in this region in association with persistence of viral infection[5]. Significant mutations in HVR-1 arise due to the presence of a specific anti-E2 humoral immune response. It is emerging that genetic heterogeneity extends throughout the entire HCV genome, and that this allows HCV infection to persist. Anti-HCV-specific T cell responses efficiently clear viruses during the acute phase of infection[6]. HVR quasispecies may have important biological consequences for the persistence of hepatitis C viral infection and the severity of liver disease[5,7].

HCV quasispecies populations result from the error rate (misincorporation frequencies averaging from about 10^{-4} to 10^{-5} per base) and the absence of proof-reading by RDRP. Most mutant particles cannot replicate, but the fittest infectious particles are selected on the basis of their replication capacities and selective pressure exerted by their interaction with host cell proteins and by the immune response[8]. Adaptive mutations in NS4B, NS5A or NS5B strongly enhance replication, but are incompatible to each other, whereas adaptive mutations in NS3 tend to be weak and cooperatively enhance replication when combined with strongly adaptive mutations[8,9]. The adaptive mutations in NS3 and NS5B map to surface residues distant from the enzyme active site, suggesting that these changes are likely to affect the interaction between non-structural protein and cellular factors[8].

Hepatitis C viral quasispecies can be detected by cloning of reverse transcription - polymerase chain reaction products, but sequencing of 15-20 independent clones is expensive and laborious, and so is not practical for a large number of clinical samples. For large numbers of clinical samples, faster methodologies like single-strand conformational polymorphism (SSCP), heteroduplex analysis (HDA) and gel shift assay (GSA) have been developed based on the differential migration of amplified DNA in acrylamide gel electrophoresis. In the present study, we used the established and reliable method of SSCP to detect quasispecies[10-13]. The mobility of mutated DNA fragments is affected by particular changes in the single-strand DNA structure; therefore, every variant migrates in a different way.

In controlled trials on the effect of interferon and ribavirin combination therapy, the independent predictive influence of virological parameters on patients with sustained virological response (SVR) raised questions about the intrinsic properties of HCV strains, like genotype and quasispecies and their effect on sensitivity and resistance to combination therapy[14-16]. The influence of viral quasispecies with variations in the hypervariable region (HVR) and NS5A ISDR (interferon-sensitivity determining region) genes on the effectiveness of interferon has been explored world wide[14,17-20]. These studies paid special attention to the importance of quasispecies as independent predictor of SVR. The cause and mechanism of HCV resistance to interferon needs to be well understood and several viral proteins may influence the therapy outcome. Gupta R *et al* reported HCV mutations in the 5' untranslated region, ISDR and PePHD region, and their influence on therapy responses in a South-Indian population. However, the influence of quasispecies in the HVR and NS5B regions on the persistence of infection and disease severity in chronic hepatitis C patients needs to be explored.

MATERIALS AND METHODS

Study design

This study enrolled 47 patients with chronic liver disease [32 with chronic active hepatitis (CAH), 9 with cirrhosis and 6 with hepatocellular carcinoma (HCC)] verified by histological examination, Child-Pugh score, and FNAC (fine needle aspiration cytology) wherever applicable, who attended medical outpatient departments (OPDs) and wards of Lok Nayak Hospital, New Delhi, India between 2003 to 2005, on the basis of pre designed performa, with all relevant information about possible risk factors, symptoms, clinical examination, detailed biochemical investigation and serology. They had a mean age of 48.4 ± 14.5 years, a male:female ratio of 25:22 and an alanine aminotransferase (ALT) value (mean ± SD) of 79.6 ± 44.6 (26-296) IU/L. Out of 47 patients, 34 (24 with CAH, 6 with cirrhosis, and 4 with HCC) were infected with virus strain of genotype 3, 11 (7 with CAH, 2 with cirrhosis, and 2 with HCC) with virus of genotype 1 and 2 (1 with CAH and 1 with cirrhosis) with virus of mixed genotype (1 + 3). The most probable risk factor for transmission was transfusion of infected blood and contaminated blood products, in 63.8% (30/47) of the patients. Out of 47 patients, 41 (32 with CAH and 9 with cirrhosis) were on combination therapy, 30 (73.17%) attained sustained virological responses and 11 (26.83%) were non-responders. However, the 6 HCC patients were not on therapy. The 41 chronic hepatitis C (CH-C) patients (32 with chronic active hepatitis and 9 with cirrhosis) were on medication (interferon-α2b 3 MU + 1000 mg ribavarin daily), having ALT $\geqslant$ 60 IU/L and HAI score $\geqslant$ 3 at baseline. The severity of liver disease was determined by histopathological grading according to the modified Knodell Score for the CAH patients and by Child-Pugh score for the cirrhosis patients[21-23]. These 41 patients were put on 48 wk of therapy or follow up. The viral load was estimated after 0 d, 4, 8, 12, 24 and 48 wk of the therapy. Patients without any detectable HCV viremia ($\leqslant \log_{10}$ 1 IU/mL) after stopping the therapy, and even after 6 mo of therapy withdrawal, were defined as responders, and, if these patients were found to show positivity 6 mo after withdrawal of therapy, they were defined as relapsers. However, the patients with detectable HCV viremia after the end of wk 48 of therapy were defined as non-responders. Liver biopsies of CAH patients and Child-Pugh scoring in cirrhosis patients were performed at baseline, and after 24 and 48 wk of therapy. The SSCP analysis of the HVR and NS5B regions was performed in all sera samples before the initiation of treatment and after 24 wk of therapy. Genotypes were also studied by RFLP for genotypic classification of these

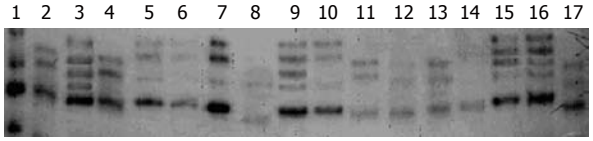


Figure 1 Representative autoradiograph of SSCP analysis of HCV RNA-positive samples showing the development of quasispecies in samples 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 15, 16 and 17.

patients (data not shown). Patients with > 4 SSCP bands were considered to have complex quasispecies (CQS) of HCV. However, the patients with 3 or 4 bands in the SSCP gel were considered to have simple quasispecies (SQS) and those with 2 bands were considered to have no quasispecies (NQS). The patients were grouped based on the number of bands in the SSCP gel (Figure 1) to study the influence of quasispecies on virological responses and disease severity. RNA extracted from sera samples (11 non-responders and 12 responders) was amplified by RT-PCR using primers specific for the HVR and NS5B genes, and the products were sequenced (Macrogen Inc, Korea) on a commercial basis at three different time (0 d, 24 wk and 48 wk) intervals during therapy in non-responders and two times (0 d and last sample before the clearance of HCV infection) in responders. Sequencing results at baseline were submitted to the GenBank database and also used for phylogeny. A phylogenetic tree was drawn to analyze the distance matrix between the SVR and non-responder (NR) patients on the basis of sequences derived from the HVR and NS5B genes, using the Bio Edit program for sequence analysis.

Biochemistry and serology

Biochemical investigations, including routine and specific liver function tests, were carried out in all cases. Serological tests were performed using commercially available ELISA kits, according to the manufacturer's instructions. Sera samples of all 47 patients were screened for HBsAg using a 3rd generation ELISA kit from Biokit, Spain; IgG HBc and HBeAg were screened using an ELISA kit from RADIM SpA, Italy; and anti-HCV antibody using a 3rd generation ELISA kit (General Biological Corporation, Taiwan). Exclusively anti-HCV-positive patients' sera were used for the detection of HCV RNA.

Reverse-transcription PCR of HVR and NS5B region

Total RNA was isolated using Tri Reagent (Sigma-Aldrich) according to the manufacturer's instructions and amplified by reverse transcription-PCR. For reverse transcription, 5 μL of extracted template RNA was added to 20 μL of reaction master mix containing reaction buffer, dNTPs, RNAsin (10 U), M-MuLV reverse transcriptase (40 units) and outer antisense primer, and incubated at 42℃ for one hour for cDNA synthesis. First-round PCR amplification was carried out using outer sense and antisense primers, 2 μL of cDNA template, reaction buffer containing magnesium, dNTPs, and Taq DNA polymerase (1 unit), in a 25-μL reaction, by denaturing at 94℃ for 5 min and 35 cycles with 60 s each of denaturation at 94℃, annealing at 55℃ and extension at 72℃, in a DNA thermal cycler (M.J. Research, USA). After the last cycle, the extension was prolonged for 8 min at 72℃. Second-round PCR was performed using 2 μL of the first PCR product as template, which was amplified with the second PCR mix containing inner primers (Table 1). The reagent composition and temperature profile were the same as those for the first PCR. Both positive and negative controls were run in parallel with the test samples. The expected PCR products for HVR and NS5B were 403 and 293 bp, respectively; these were detected in ethidium bromide-stained 30 g/L Nusieve agarose gels in a UV Transilluminator.

Single-strand conformational polymorphism

Briefly, single-strand conformational polymorphism (SSCP) was performed by radiolabelling the PCR products for an additional 10 cycles where dCTP was replaced with (α-p^{32}) dCTP (specific activity, 4000 CI/nmol) in the PCR mix. One μL of radiolabelled PCR product was diluted with 9 volumes (10 times) of denaturing solution (950 g/L formamide, 20 mmol/L EDTA pH 8.0, 0.5 g/L xylene cyanol and 0.5 g/L bromophenol blue), heat-denatured for 5 min at 95℃, and chilled on ice for 3 min; 3 μL of this diluted radiolabel product was subjected to non-denaturing electrophoresis in a 60 g/L polyacrylamide gel containing 100 mL/L glycerol. Gels were run in 1 × TBE for 16 h at 250 V in a DNA sequencing apparatus, at 17 ± 1℃, dried on a gel drier, and exposed to X-ray film with an intensifying screen at -20℃. Electrophoretic mobility shifts of single-strand DNA bands were analyzed following development of the X-ray film.

Hepatitis C viral quantitation by real time PCR

Total RNA was used for real time quantification. The RealArt HCV PCR Reagents constitute a ready-to use system for PCR amplification and the detection of HCV RNA on the Light Cycler instrument (Rotor Gene 3000, Corbett Research Inc.). The RealArt HCV PCR master mixture contains reagents and enzymes for the amplification of a 240-bp region of the HCV genome with the parallel detection (A.Ch 1-FAM) and quantification of the amplified PCR products in comparison to five standard samples diluted 10-fold, which were used to generate the standard curve.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test, ANOVA, Chi-square, and Fischer's exact test, wherever applicable, using the software EPISTAT and SPSS. Statistical analysis was performed by comparisons of two groups descriptively. Patients were classified as being infected with complex quasispecies (CQS), simple quasispecies (SQS) and no quasispecies (NQS) on the basis of their SSCP pattern, representing the level of HCV quasispecies variations. The results are expressed as means ± SD. $P < 0.05$ was considered significant.

RESULTS

Quasispecies

Patients were screened for the presence of quasispecies by SSCP analysis, on the basis of differential migration in PAGE

Table 1 Primers used for reverse transcription-PCR of the HVR and NS5B genes of HCV

Region/Nucleotide position	Primer set	Forward/Reverse	Sequence (5'-3')	Amplicon size (bp)
HVR	Outer	Forward	GTGGTTTGGATGTGTGTGGATGAAC	
2040-2443		Reverse	TATTGCACGTCCACGATGTTCTG	403
	Inner	Forward	GGCTCCCCCTTGCAACATCT	
		Reverse	GTGGAGGTGTATGAGCCCTGTTG	
NS5B	Outer	Forward	TGGGGATCCCGTATGATACCCGCTGCTTTGA	
8301-8593		Reverse	GGCGGAAT (T/A) CCTGGTCATAGCTCCGTGAA	293
	Inner	Forward	CTCAAC (C/T) GTCACTGAACAGGACAT	
		Reverse	CCACGAC (T/C) AGATC (A/G) TCTCCG	

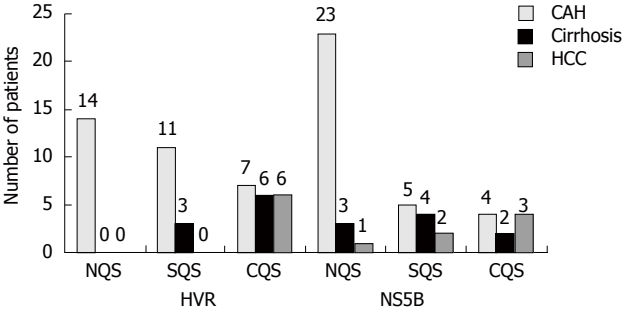


Figure 2 Patients with chronic active hepatitis, cirrhosis and HCC with different degrees of quasispecies with variations in the HVR and NS5B genes of HCV, observed by SSCP.

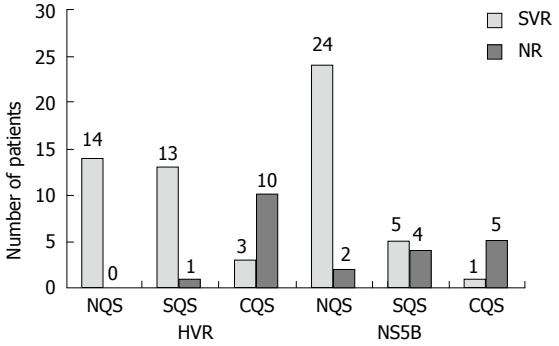


Figure 3 Virological responses in patients with different degrees of quasispecies with variations in the HVR and NS5B genes of HCV, observed by SSCP.

denaturing gels. The appearance of more than 2 bands in gels is associated with the presence of quasispecies. Patients with more than 4 bands in SSCP gels were infected with complex quasispecies (CQS). Complex quasispecies were detected both in the NS5B and HVR regions of HCV, but were relatively more abundant in the HVR (n = 19) than the NS5B (n = 9) region. Among the 32 patients with chronic active hepatitis, 18 (56.3%) showed presence of quasispecies (CQS-7, SQS-11, NQS-14) when the HVR gene was used for the analysis, while 9 (28.2%) showed presence of quasispecies (CQS-4, SQS-5, NQS-23) when the NS5B gene was used for the analysis. All 9 cirrhosis patients showed presence of quasispecies (CQS-6, SQS-3) when the HVR gene was used for the analysis, while only 6 (66.7%) showed presence of quasispecies (CQS-2, SQS-4, NQS-3) when the NS5B gene was used (Figure 2). It was interesting to note that all 6 HCC patients were positive for complex quasispecies when the HVR was used for the analysis, while 5 out of the 6 (83.3%) showed presence of quasispecies (CQS-3, SQS-2, NQS-1) on the basis of the NS5B gene (Figure 2). These results showed the influence of the occurrence of HVR quasispecies on the disease severity in hepatitis C infected patients as all cirrhosis and HCC patients showed evolution of quasispecies in this region. However, NS5B quasispecies also showed their influence on disease severity. No difference was observed in the SSCP band patterns of the patients after 24 wk of therapy, confirming the accuracy of the PCR reaction and SSCP profiles. The genotypic classification of quasispecies was not feasible from this data due to sample size. However, the occurrence of quasispecies in both the genotypes (genotypes 1 and 3) was observed.

Virological responses

Among the 41 patients on therapy, 30 had sustained virological responses, whereas 11 were non-responders. HVR quasispecies were detected in 27 out of 41 (65.9%) patients (CQS-13, SQS-14, NQS-14), while NS5B quasispecies were detected in 15 out of 41 (36.6%) (CQS-6, SQS-9, NQS-26) (Figure 3). Out of the 11 non-responders, 10 were infected with complex quasispecies (> 4 bands in the SSCP) and 1 was infected with simple quasispecies, based on analysis of the HVR gene; when the NS5B gene was analyzed, among the 11 non-responders, 5 were infected with complex quasispecies, 4 with simple quasispecies and 2 with no quasispecies. Among the 30 SVR patients, 16 (53.3%) were infected with HVR quasispecies (CQS-3, SQS-13, NQS-14), whereas 6 (20.0%) were infected with NS5B quasispecies (CQS-1, SQS-5, NQS-24) (Figure 3). These results suggest that the presence of complex quasispecies with variations in the HVR gene has an impact on virological response, as 90.9% (10/11) of patients infected with complex quasispecies with variations in this region did not respond to combination therapy, whereas only 10.0% (3/30) of patients infected with complex quasispecies were responders.

Correlation of viral kinetics with quasispecies

Out of 41 patients on therapy, HVR quasispecies were seen in 27 patients. No statistical difference was observed in viral load of patients with CQS, SQS and NQS (mean ± SD $\log_{10}$ IU/mL) from baseline (CQS-6.9 ± 0.29, SQS-6.8 ± 0.26, and NQS-6.9 ± 0.22) to 8 wk (CQS-4.3 ± 1.05, SQS-4.5 ± 1.2, NQS-4.1 ± 1.1) of combination therapy with minimum standard error. A statistically significant

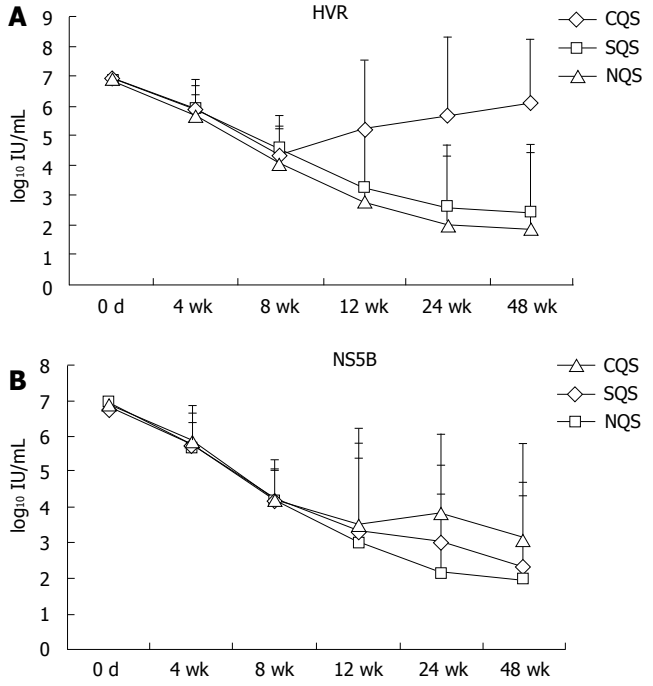


Figure 4 Viral load (mean ± SD) at different phases during 48 wk of therapy in patients grouped on the basis of the number of bands observed in the selected region. **A**: Viral kinetics of patients grouped on the basis of quasispecies in the HVR region (CQS-13, SQS-14, NQS-14); **B**: Viral kinetics of the patients grouped on the basis of quasispecies in the NS5B region (CQS-6, SQS-9, NQS-26) by SSCP analysis.

difference in the viral load ($P < 0.05$; CQS *vs* SQS, NQS) was observed after 12 wk (CQS-5.2 ± 2.3, SQS-3.2 ± 1.9, NQS-2.8 ± 2.4) of therapy in patients with HVR region quasispecies of HCV. A statistically significant difference in viral load ($P < 0.05$) with high standard error was observed in comparisons of CQS with SQS and NQS after 24 wk (CQS-5.7 ± 2.6, SQS-2.6 ± 2.1, NQS-2.0 ± 2.33) and 48 wk (CQS-6.1 ± 2.1, SQS-2.4 ± 2.3, NQS-1.9 ± 2.5) of therapy (Figure 4A). No significant difference ($P > 0.05$) was observed in the mean viral load (log_{10} IU/mL) from baseline (CQS-6.9 ± 0.29, SQS-6.8 ± 0.3, NQS-6.94 ± 0.22) to 12 wk (CQS-3.5 ± 2.7, SQS-3.3 ± 2.5, NQS-3 ± 2.4) in comparisons of patients with different levels quasispecies (CQS, SQS and NQS) in NS5B region. However, the standard error was almost negligible at baseline, but an increased standard error in viral load was observed after 12 wk of therapy. A statistically significant difference ($P < 0.05$) with high standard error was observed between the mean viral loads of patients infected with CQS and those infected with NQS after 24 wk (CQS-3.9 ± 2.2, SQS-3.0 ± 2.2, and NQS-2.1 ± 2.3) and 48 wk (CQS-3.1 ± 2.7, SQS-2.3 ± 2.4, NQS-2.0 ± 2.3) of therapy (Figure 4B). A continuous decline in mean viral load was observed in patients without complex quasispecies infection, suggestive of an influence of the presence of quasispecies on viral kinetics and therapeutic outcome during combination therapy.

Correlation of disease severity with the presence of quasispecies

The histological activity index (HAI) score is an important factor for the assessment of disease severity in CAH patients. It was interesting to note that mean HAI scoring (mean ± SD) was comparable among groups based on the degree of quasispecies diversity in the HVR at baseline (CQS-6.64 ± 2.9, SQS-6.72 ± 1.2, NQS-6.9 ± 0.22). Patients grouped on the basis of genetic variability in the NS5B gene showed a statistically significant difference (CQS *vs* NQS) in the mean HAI scores at baseline (CQS-7.6 ± 3.3, SQS-6.7 ± 2.2, NQS-5.86 ± 2.08). An improvement in the severity of disease was observed in 80.0% (24/30) of the SVR (sustained virological response) patients. Bridging of fibrosis was observed in all NR (non-responders) and 13.3% (4/30) of the SVR patients. No statistical significant difference in the results of liver function tests was observed among patients with different degrees of HVR and NS5B quasispecies, at baseline and on follow-up every 4 wk until 48 wk.

Quasispecies and phylogeny

Amino acid variation in the NS5B gene plays a major role in the replication of the virus and may be important for the virological response during combination therapy. It was interesting to note that the number of mutations was higher in genotype 1 as compared to genotype 3 in the HVR region, suggesting an influence of genotype on the rate of mutations in the selected region of HCV and on the evolution of quasispecies. Amino acid variations in the HVR and NS5B regions of different genotypes among patients (n = 23, Genotype 1 = 5, Genotype 3 = 18) during different phases of therapy are suggestive of the occurrence of mutations during the course of therapy. The sequences of virus strains during the course of therapy showed 2-5 amino acid substitutions and synonymous mutations at the nucleotide level in both HVR and NS5B regions, compared with the sequences of virus strains before therapy (data not shown as no specific mutation was observed to be responsible for the persistence of infection during therapy). Phylogenetic analysis of sequenced virus isolated from patient samples showed different distance patterns between responders and non-responders in HVR and NS5B regions, as shown in Figures 5 and 6. The non-responders were on a different branch, suggestive of no close pairing between these non-responder strains of genotype 3 in the HVR region. However, closing pairing was observed between non-responder strains of genotype 3 in the NS5B region: 5 non responder patients were on the same branch and 3 patients were on the other branch. However, all four non-responder were on the same branch in patients of genotype 1. Strains appearing on the same branch (close pairing) during phylogenetic tree analysis possess significant homology in their nucleotide sequences. These results suggest that either the selection of resistant strains or the development of quasispecies may be the cause of unresponsiveness to combination therapy in HCV patients; however, these mechanisms require further analysis.

DISCUSSION

Diversifications in the HCV genome are the results of viral RNA polymerase infidelity, immune-driven selection and exertion of therapy pressure or a combination of these processes. PCR-SSCP analysis is a well established

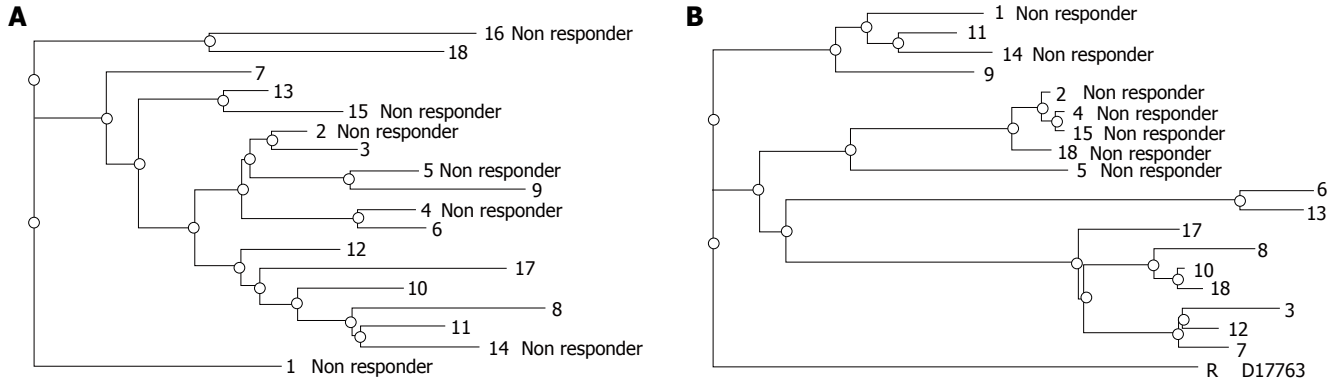


Figure 5 Phylogenetic analysis of the 18 (7 NR and 11 SVR) patients infected with HCV of genotype 3 by the neighbor-joining method in order to draw a distance matrix of the sequences. **A**: HVR, the accession numbers of the HVR genes in patients 1-18 are EF015726 to EF015743 in order; **B**: NS5B region, the accession numbers of the NS5B regions in patients 1-18 are EF015699 to EF015716 in order.

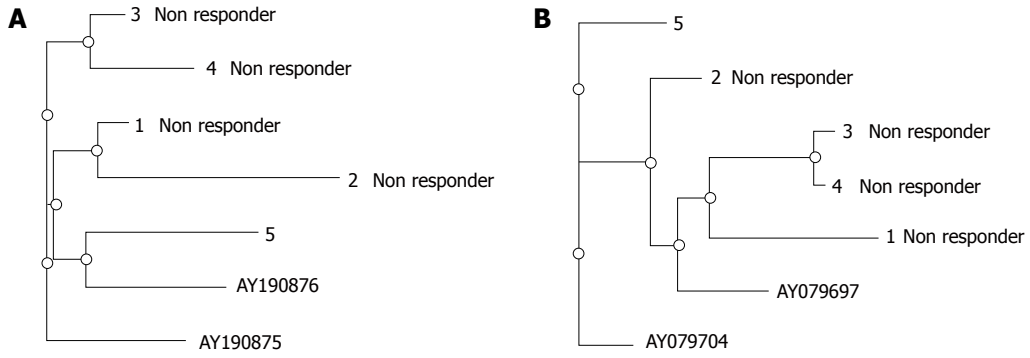


Figure 6 Phylogenetic analysis of the 5 (4 NR and 1 SVR) patients infected with HCV of genotype 1 by the neighbor-joining method, in order to draw a distance matrix of the sequences. **A**: HVR, the accession numbers of the HVR gene in patients 1-5 are EF015744 to EF015748 in order; **B**: NS5B region, the accession numbers of the NS5B region in patients 1-5 are EF015721 to EF015725 in order.

technique to study genetic variability, including the presence of mutations and quasispecies[10-13]. Millions of viruses are produced *via* replication and eliminated each day due to exertion of immune pressure and interferon therapy[16]. Ribavirin does not have any effect on the viremia level of HCV-infected patients, but it reduces the ALT level, increases the likelihood of sustained virological response and reduces the probability of relapse[16].

The HVR and NS5B genes were selected in the present study because the persistence of infection may be associated with positive selection of HVR quasispecies by the host immune system and because of the major role of NS5B in the replication of hepatitis C. Factors like age, sex and duration of infection may be possible causes of the occurrence of quasispecies; in this study, we studied virological responses in patients infected with quasispecies before the initiation of therapy and looked for alterations in the SSCP patterns during therapy. Several conflicting reports about a possible correlation of therapy response and disease severity with the presence of quasispecies have been published[15,20,24-27]. The genes encoding HVR protein exhibit a high degree of variability, giving rise to differing phenotypic traits, including alterations in receptor-binding affinity and escape from immune recognition[28]. Substitutions in the NS5B gene of HCV may correlate with the virological response to interferon-based therapy[29-31].

The occurrence of quasispecies was proportionately higher in patients with HCC and cirrhosis than in CAH patients, revealing a significant correlation between the molecular evolution of quasispecies and the severity of disease in hepatitis C patients in this study. The genetic diversity at baseline can be predictive of distinct virological behavior during the course of combination therapy. Among the 13 patients infected with complex quasispecies with variations in the HVR gene, 10 were non-responders at baseline. This correlates significantly with the persistent of infection as the response rate against interferon may be due to two mutually compatible approaches; the infecting virus might have become naturally resistant to interferon or the virus could have evolved into quasispecies. The non-responders had normal signal transduction pathways required for the intracellular activity of interferon[32]. The recognition of the HVR epitope (envelope) by immune cells and the exertion of interferon-based therapy play a major role in the evolution of quasispecies.

Several studies have shown an association of epidemiological factors and treatment regime with the presence of quasispecies[13,14,19,33]. Complex quasispecies with variations in the HVR and NS5B genes of HCV were significantly associated with virological response during wk 48 of therapy. Virus quasispecies evolution within patients increases the pathogenicity of HCV. The resulting viral quasispecies are heterogeneous and sensitive to selection pressures. Complex variants escape from immune pressure and the antiviral spectrum of the combination therapy[34]. No mutant strains have the capacity for replication, but only selective mutants can replicate, which may be responsible for persistence of infection. The circulating viremia in blood and liver cells is significantly associated with disease prognosis[35]. There was no statistically significance difference in the HAI scores among patients with different degrees of HVR quasispecies at baseline. However, there was a

significant difference in the HAI scores at baseline between patients infected with different genetic variants (CQS *vs* NQS) of the NS5B gene. Selective adaptive mutations in the resistant strains occurring during replication influence the RDRP activity and lead to increases in viremia level and HAI scoring[7-9]. These results suggest the significance of evolution of quasispecies on virological responses and disease severity.

The amino acid variability in HVR and NS5B was evaluated from cDNA-derived sequences. The occurrence of HVR quasispecies influences the therapeutic outcome; because no strains isolated from non-responder patients showed close pairing, it is possible that some other factors may be responsible for the persistence of infection. However, the possibility of resistant strains can not be denied. The NS5B sequences of non-responder strains were on the same branch, showing the possibility of resistant strain infection in these patients. However, adaptive mutations in NS5B lead to persistence of infection and a severe course of liver disease in patients. These findings reveal that the occurrence of HVR quasispecies, including the presence of resistant strains, is a major factor influencing the therapeutic outcome and disease severity in chronic hepatitis C patients. Genetic variability was not uniformly distributed throughout the HCV genome.

In conclusion, viral quasispecies heterogeneity can be an important marker to predict the response of combination therapy in patients with chronic hepatitis C. Quasispecies complexity on the basis of SSCP pattern could be helpful in differentiation of SVR and NR patients. Complex quasispecies heterogeneity causes high HCV viremia, which was significantly correlated with the severity of liver disease and prognosis during the course of combination therapy. However, there was no significant alteration in SSCP profile during therapy. The occurrence of resistant strains may be another factor contributing to the persistence of HCV infection. Quasispecies in both HVR and NS5B genes may have a major impact on treatment and be a good predictor of virological response. Complex quasispecies are resistant to combination therapy and influence disease prognosis during therapy. Further evaluation of viral quasispecies complexity in the HVR and NS5B genes, as well as other HCV genes (whole genome), is required for a better understanding of the influence of quasispecies on the persistence of infection and disease prognosis during the course of therapy in HCV-infected patients.

COMMENTS

Background

The hepatitis C virus (HCV) continues to persist unless patients are put on interferon therapy. Treatment with interferon-based therapy and error prone replication of the viral genome *via* RNA-dependent RNA polymerase (RDRP) accelerates the evolution of HCV variants during the course of infection. The highly heterogeneous population in HCV-infected patients, consisting of quasispecies, developed because of interferon therapy, immune pressure and replication strategy. Quasispecies are one of the major virological factors other than genotypes that can influence the response to therapy in chronic hepatitis C patients; their role in this needs to be evaluated.

Research frontiers

HCV quasispecies can be detected by cloning of reverse transcription-polymerase chain reaction products; however, sequencing of 15-20 independent clones is expensive and laborious, and not practical for a large number of clinical samples. For large numbers of clinical samples, faster methodologies like single-strand conformational polymorphism (SSCP), heteroduplex analysis (HDA) and gel shift assay (GSA) have been developed based on the differential migration of amplified DNA in acrylamide gel electrophoresis. In the present study, we used the established and reliable method of SSCP for the detection of quasispecies with variations in the HVR and NS5B genes. The patients were grouped on the basis of the number of bands in the SSCP gel. Patients with > 4 SSCP bands were considered to be infected with complex quasispecies (CQS) of HCV. Patients with 3 or 4 bands were considered to be infected with simple quasispecies (SQS); and those with 2 bands were considered to be infected with no quasispecies (NQS). Our interest was to evaluate whether or not the occurrence of quasispecies at baseline influences the virological response and disease severity in chronic hepatitis C patients.

Innovations and breakthroughs

The significance of disease severity on the occurrence of HVR quasispecies in serum of hepatitis C infected patients was observed as all of the cirrhosis and HCC patients showed evolution of quasispecies in this region. However, a major role of NS5B quasispecies was also observed with regard to disease severity. No difference was observed in the SSCP patterns of patients after 24 wk of therapy, confirming the validity of the PCR reaction and SSCP profiles. These results suggest an effect of complex quasispecies with variations in the HVR gene on virological response, as 90.9% (10/11) of patients infected with complex quasispecies with variations in this region did not respond to combination therapy, whereas only 10.0% (3/30) patients infected with complex quasispecies showed SVR. A continuous decline of mean viral load was observed in the patients without complex quasispecies viral infection, suggestive of an influence of quasispecies on viral kinetics and therapeutic outcome during combination therapy.

Applications

The study suggests that quasispecies complexity in both HVR and NS5B genes had a major impact on treatment and is a good predictor of virological responses. However, the existence of quasispecies based on other HCV genes should be further evaluated for a better understanding of the influence of quasispecies on virological responses and disease severity in patients with chronic hepatitis C.

Terminology

Chronic active hepatitis (CAH): anorexia, weight loss, jaundice, ascites, edema present singly or in combination, persistently or intermittently and biochemical features (SGOT, SGPT more than twice normal and globulin 1.5 times normal) of hepatocellular disease for more than 6 months duration. Liver biopsy is necessary for the assessment of disease severity. A diagnosis of cirrhosis of the liver was considered on the basis of the following criteria: ascites, splenomegaly, shrunken liver, endoscopy showing esophageal varices and biochemical features of hepatocellular failure characterized by low serum albumin level (< 2.5 gm). Hepatocellular Carcinoma (HCC): patients were evaluated on the basis of history, clinical examination, liver function tests and serological tests for hepatitis B & C, serum alpha-feto protein levels and liver imaging [ultrasound sonography (US), computed tomography (CT), magnetic resonance imaging (MRI)], and final diagnosis of HCC was made on FNAC (fine needle aspiration cytology). Quasispecies: The origination of a highly heterogeneous population in hepatitis C-infected patients as a result of interferon therapy, immune pressure and replication strategies.

Peer review

In this study the authors assessed whether the occurrence of HCV quasispecies influence responses to antiviral therapy and the severity of chronic hepatitis C. Quasispecies were assessed on the basis of SSCP patterns. The results are interesting and suggest that complex quasispecies are a major factor in non-response to combination therapy. The SSCP pattern remains unaltered during the course of therapy. The concept of this study is interesting; evaluation of other HCV genes should be performed in future work.

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RAPID COMMUNICATION

Increased prevalence of symptoms of gastroesophageal reflux diseases in type 2 diabetics with neuropathy

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Abstract

AIM: To analyze the prevalence of gastroesophageal reflux disease (GERD) related symptoms in patients with diabetes mellitus (DM) and to find out the relationship between diabetic neuropathy and the prevalence of GERD symptoms.

METHODS: In this prospective questionnaire study, 150 consecutive type 2 diabetic patients attending the endocrine clinic were enrolled. A junior physician helped the patients to understand the questions. Patients were asked about the presence of five most frequent symptoms of GERD that included heartburn (at least 1/wk), regurgitation, chest pain, hoarseness of voice and chronic cough. Patients with past medical history of angina, COPD, asthma, cough due to ACEI or preexisting GERD prior to onset of diabetes and apparent psychiatric disorders were excluded from the survey. We further divided the patients into two groups based on presence or absence of peripheral neuropathy. Out of 150 patients, 46 had neuropathy, whereas 104 patients did not have neuropathy. Data are expressed as mean ± SD, and number of patients in each category and percentage of total patients in that group. Normal distributions between groups were compared with Student *t* test and the prevalence rates between groups were compared with Chi-square tests for significance.

RESULTS: The average duration of diabetes were 12 ± 9.2 years and the average HbA1c level of this group was 7.7% ± 2.0%. The mean weight and BMI were 198 ± 54 lbs. and 32 ± 7.2 kg/m^2. Forty percent (61/150) patients reported having at least one of the symptoms of GERD and thirty percent (45/150) reported having heartburn at least once a week. The prevalence of GERD symptoms is higher in patients with neuropathy than patients without neuropathy (58.7% *vs* 32.7%, $P < 0.01$). The prevalence of heartburn, chest pain and chronic cough are also higher in patients with neuropathy than in patients without neuropathy (43.5% *vs* 24%; 10.9% *vs* 4.8% and 17.8% *vs* 6.7% respectively, $P < 0.05$).

CONCLUSION: The prevalence of GERD symptoms in type 2 DM is higher than in the general population. Our data suggest that DM neuropathy may be an important associated factor for developing GERD symptoms.

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Key words: Diabetes; Neuropathy; Gastroesophageal reflux disease; Symptom; Heart burn

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INTRODUCTION

The prevalence of gastrointestinal (GI) symptoms is reported to be higher in patients with diabetes mellitus (DM)[1] than the general population. Although not generally considered as important causes of morbidity or mortality in DM patients, these symptoms can influence health related quality of life (HRQOL) and affect productivity and employment status[2,3]. GI symptoms can be encountered in up to 75% of diabetic outpatients evaluated in tertiary care referral centers[4,5]. Many patients with GI disorders remain undiagnosed and untreated. The underlying mechanisms of the high prevalence of gastrointestinal symptoms in type 2 DM are poorly defined and controversy exists at the present time. The association of GI symptoms in DM to glycemic control is debated and controversy exists[6,7]. Many of the gastrointestinal symptoms in diabetics suggest motor dysfunction, and a neuropathic basis to those dysfunctions would be a reasonable explanation. A recent study by Bytzer *et al* suggests that GI symptoms in DM were associated with complications of DM, particularly peripheral neuropathy[6]. Some studies even suggest that gastrointestinal symptoms in

diabetic patients were poorly related to diabetic neuropathy, instead were related with psychiatric illness[8,9]. The duration of DM was also an important factor for GI symptoms in type 2 DM patients since longer duration tends to have more complications[1,10].

In the pathophysiology of gastroesophageal reflux disease (GERD), a major mechanism that promotes reflux of gastric contents into esophagus is delayed gastric emptying, a frequent complication of diabetes with neuropathy. The most common symptoms of GERD are heartburn and regurgitation[11] and GERD may also responsible for other symptoms such as hoarseness, asthma (chronic cough) and chest pain. The aim of this study is to analyze the prevalence of GERD symptoms in patients with diabetes and to find out the relationship between the duration of diabetes, BMI, HbA1c, diabetic neuropathy and the prevalence of GERD symptoms. We especially focus on the role of DM neuropathy in the development of symptoms of GERD in type 2 diabetes.

MATERIALS AND METHODS

In this cross-sectional study, 150 type 2 diabetic patients were enrolled. This was comprised of 150 consecutive type 2 diabetic patients attending the endocrine clinic in a major University diabetic center. This was elicited based on a questionnaire distributed to patients.

A junior physician helped the patients to understand the questions. Patients were asked about the presence of five most frequent symptoms of GERD that included heartburn, regurgitation, chest pain, hoarseness of voice and chronic cough[11]. To maximize the specificity of symptoms of GERD for our analysis, we considered only participants with frequent symptoms and excluded patients with less frequent than weekly. Patients' weight, height, BMI, BP, duration of diabetes, medications, HgbA1c and complications of diabetes including neuropathy were obtained within 6 wk of eliciting the answers to the questionnaire. Patients with past medical history of angina, COPD, asthma, cough due to ACEI or preexisting GERD prior to onset of diabetes and apparent psychiatric disorders were excluded from the survey.

We further divided the patients into two groups based on presence or absence of peripheral neuropathy. Out of 150 patients, 46 had neuropathy, whereas 104 patients did not have neuropathy. Presence of neuropathy was determined by past change in neuropathy and positive sensory symptoms including: limbs numbness, prickling sensation, aching pain, burning pain and at least one of the positive neurological examination findings including: (1) decreased pressure or pain sensation: positive monofilament test; (2) decrease of light touch sensation: positive cottonwool swab test; (3) decreased tendon refluxes.

Data are expressed as mean ± SD, and number of patients in each category and percentage of total patients in that group. Normal distributions between groups were compared with Student *t* test and the prevalence rates between groups were compared with Chi-square tests for significance. $P < 0.05$ is selected as significant level.

Table 1 Prevalence of GERD symptoms in DM patients with and without neuropathy

	DM 150	DM w Neuro 46	DM w/o Neuro 104	*P* value
Age (yr)	54 ± 15	56 ± 13	53 ± 16	> 0.10
DM Duration (yr)	12 ± 9.2	14.8 ± 9.1	10.7 ± 9.0	> 0.10
Weight (lbs)	198 ± 54	207 ± 42	194 ± 58	> 0.10
BMI	32 ± 7.2	33.9 ± 6.9	31.2 ± 7.2	> 0.10
HbA1c (%)	7.7 ± 2.0	7.9 ± 1.8	7.7 ± 1.2	> 0.10
Metformin	87/150 (58%)	26/46 (56.5%)	61/104 (58.6%)	> 0.10
GERD	61/150 (40.7%)	27/46 (58.7%)	34/104 (32.7%)	< 0.01
Heartburn	45/150 (30%)	20/46 (43.5%)	25/104 (24%)	< 0.05
Chest pain	10/150 (6.6%)	5/46 (10.9%)	5/104 (4.8%)	< 0.05
Chronic cough	14/150 (9.4%)	8/46 (17.8%)	7/104 (6.7%)	< 0.05
Regurgitation	19/150 (12.8%)	7/46 (15.6%)	12/104 (11.5%)	> 0.10
Hoarseness	5/150 (3.3%)	2/46 (4.4%)	3/104 (2.9%)	> 0.10

GERD: Presence of one or more of the five symptoms. Prevalence of heartburn in US adults is 14% weekly[11].

RESULTS

The mean age ± SD of the group was 54 ± 15; the mean weight and BMI were 198 ± 54 lbs. and 32 ± 7.2 kg/m^2. Eighty-seven (58%) of the patients were women. Ninety two (61%) patients were Caucasians, twenty-five (16.7%) of the patients were African Americans, seventeen (11.3%) of the patients were Asia Americans and twenty-one (14%) patients were from the other ethnic groups. The average duration of diabetes was 12 ± 9.2 years and the average HbA1c level of this group was 7.7% ± 2.0%. All patients took multiple medications including eighty-seven patients (56.5%) took metformin for their DM control.

Forty percent (61/150) patients reported having at least one of the symptoms of GERD and 30% (45/150) reported having heartburn at least once a week (Table 1).

There is no difference in age, body weight, BMI, duration of DM, and combination therapy with metformin between patients with neuropathy and patients without neuropathy ($P > 0.05$). The prevalence of GERD symptoms is higher in patients with neuropathy than patients without neuropathy ($P < 0.01$). The prevalence of heartburn, chest pain and chronic cough are also higher in patients with neuropathy than patients without neuropathy ($P < 0.05$) (Table 1).

DISCUSSION

Gastrointestinal symptoms are frequently encountered in patients with DM. Although not generally considered important causes of mortality in DM patients, these symptoms can influence Health Related Quality of Life (HRQOL)[2,3] and be encountered in up to 75% of diabetic outpatients[4,5]. Our data showed that the overall prevalence of GERD symptoms in diabetics is 40.7% and is greater than in general population (14%) reported in the literature[12]. In a study by Nebel *et al* they reported 7% had daily heartburn and 14% had weekly symptoms[12]. In those with neuropathy, the presence of heartburn (43.5%) was significantly higher than in the group without neuropathy (24%; $P < 0.05$). The mechanisms of the high

prevalence of gastrointestinal symptoms in type 2 DM are poorly defined and controversy at present time. The proposal mechanisms include: over weight and obesity, blood glucose levels at the time of presentation, DM medications, duration of DM and DM complications.

Over weight and obesity are risk factors for symptoms of GERD[13-15], and recent study by Jacobson *et al* found BMI is associated with symptoms of GERD in both normal-weight and overweight women[16]. Most of our type 2 diabetes patients are over weight or obesity with average BMI of 32, so over weight might be explained part of high frequent symptoms of GERD in type 2 diabetes patients. However, there was no difference in BMI between patient with or without neuropathy, over weight or obesity might not associate with the high frequency of GERD symptoms of type 2 DM patients with neuropathy. Acute hyperglycemia (> 200 mg/dL) is a contributory factor for the GI symptoms that can slow gastric motor function and delay gastric empty. Bytzer and colleagues reported that gastrointestinal symptoms were associated with DM complications but not with current glycemia control measured by blood glucose and glycosylated hemoglobin levels and a later study from the same group suggested that GI symptoms were associated with both poor glycemic control and DM complications[1]. Our data indicate that the symptoms of GERD were not associated with recent HbA1c levels. DM medications, especially metformin induce many gastrointestinal symptoms including nausea, vomiting, diarrhea, flatulence, chest discomfort and abdomen discomfort but not heartburn. Since there was no difference in medications usage between patients with neuropathy and without neuropathy, medications might not associate with the high prevalence of GERD symptoms in type 2 DM patients with neuropathy.

In our study, we focused on the symptoms of GERD and found that the prevalence of GERD symptoms is higher in patients with neuropathy (58.7%) than in those without neuropathy (32.7%). Heartburn is noted in 42% of patients with neuropathy *versus* 24% patients without neuropathy. Since experience of heartburn is likely to be blunted by neuropathy, the actual incidence of GERD may even be higher. Our data thus suggest that neuropathy is an important associate factor of the symptoms of GERD in type 2 DM. Delayed gastric emptying is a well-known mechanism promoting gastric retention and reflux. In type 1 DM, peripheral neuropathy is present in the majority of patients with enteropathy and GI symptoms are associated with DM peripheral neuropathy[1]. Two recent repots suggest that the pacemaker cells in the wall of the upper digestive tract are abnormal in experimental diabetes and in a single DM patient[17,18]. It is generally believed that gastroparesis of some degree complicates diabetes of > 10 years in duration when accompanied by peripheral and autonomic neuropathy. Type 2 diabetics with GI symptoms most commonly presented with gastroesophageal reflux disease whereas type 1 diabetics had bloating fullness and satiety[7].

Ideally all patients above the age of 50 and those with alarm symptoms (dysphagia, weight loss, anemia, history of cigarette smoking and alcoholism) with GERD symptoms should undergo esophago gastro duodenoscopy, and gastric emptying studies. However we did not perform these studies since our goal is not to identify a pathogenic mechanism but first to note whether neuropathy contributed to GERD symptoms. Several studies have been published that the validity and reliability these symptoms in identify cases of GERD [13,14]. Furthermore, to improve our specificity for GERD, we restricted our primary analysis to patients who reported at least weekly symptoms.

In summary, the prevalence of GERD symptoms in type 2 DM is higher than in general population and the prevalence of GERD symptoms is higher in patients with neuropathy than patients without neuropathy. Our data suggest that DM neuropathy is an important associated factor for developing GERD symptoms. Patients with DM and neuropathy should be presumed to have GI abnormalities until proven otherwise. The work up should start with thorough patients' history, physical examination, appropriate laboratory test and possible endoscopy examination. Appropriate therapy including dietary manipulation and pharmacological therapy should be given to patients with GERD symptoms to improve health related quality of life.

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COMMENTS

Background

Many investigators have reported that the prevalence of gastrointestinal (GI) symptoms is higher in patients with diabetes mellitus (DM) than the general population. Although not generally considered as important causes of morbidity or mortality in DM patients, these symptoms can influence health related quality of life and affect productivity and employment status. The underlying mechanisms of the high prevalence of gastrointestinal symptoms in type 2 DM are poorly defined. The proposal mechanisms include: over weight and obesity, blood glucose levels at the time of presentation, DM medications, duration of DM and DM complications. The association between diabetes peripheral neuropathy and GERD symptoms is controversy at present time. In the present study, we focus on the association of DM neuropathy and the symptoms of gastroesophageal reflux disease (GERD) in type 2 diabetes.

Research frontiers

To analyze the prevalence of GERD symptoms in patients with diabetes and to find out the relationship between the duration of diabetes, BMI, HbA1c, diabetic neuropathy and the prevalence of GERD symptoms. We especially focus on the association between DM neuropathy and symptoms of GERD in type 2 diabetes.

Innovations and breakthroughs

In our study, we found in first time that the prevalence of GERD symptoms is higher in patients with neuropathy (58.7%) than in those without neuropathy (32.7%). Heartburn is noted in 42% of patients with neuropathy *versus* 24% patients without neuropathy. Since experience of heartburn is likely to be blunted by neuropathy the actual incidence of GERD may even be higher.

Applications

Our data suggest that DM neuropathy is an important associated factor for developing GERD symptoms. Patients with DM and neuropathy should be presumed to have GI abnormalities until proven otherwise. The work up should start and appropriate therapy should be given to patients with GERD symptoms to improve health related quality of life.

Peer review

This is a very good study. Much more solid and valid conclusions could be drawn if more appropriate statistical analysis were done.

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RAPID COMMUNICATION

Chemokine system polymorphisms, survival and hepatocellular carcinoma occurrence in patients with hepatitis C virus-related cirrhosis

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Abstract

AIM: To explore the influence of polymorphisms in genes encoding for the chemokines Stromal cell-Derived Factor-1 *(SDF-1)/CXCL12* and Monocyte Chemotactic Protein-1 *(MCP-1)/CCL2*, or for the chemokine receptor *CCR5* on the risks of liver-related death and hepatocellular carcinoma (HCC) occurrence in hepatitis C virus (HCV)-infected patients.

METHODS: *SDF-1 3'A*, *MCP-1 (-2518)* and *CCR5-Δ32* polymorphisms, SDF-1α, Regulated upon Activation Normal T cells Expressed and Secreted (RANTES)/CCL5 and MCP-1 serum levels were determined in 120 HCV-infected patients, included at time of cirrhosis diagnosis and prospectively followed-up.

RESULTS: During follow-up, 23/120 (19.1%) patients died and 47/120 (39.1%) developed HCC. Carriers and noncarriers of each genetic marker had similar baseline characteristics estimating the severity of liver disease. The occurrence of death or HCC during follow-up was similar among carriers and noncarriers of each polymorphism. There was no association between the carriage of mutated alleles and chemokine serum levels and the latter were not associated with the risks of death or HCC.

CONCLUSION: This study suggests the lack of association of *SDF-1 3'A*, *MCP-1 (-2518)*, *CCR5-Δ32* polymorphisms with death and HCC occurrence in cirrhotic HCV-infected patients.

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Key words: Chemokine; Polymorphism; Cirrhosis; Hepatocarcinoma; Hepatitis C virus

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INTRODUCTION

Host factors influencing the course of hepatitis C virus (HCV)-related liver disease are still poorly understood. As HCV infection is a major health issue worldwide leading to life-threatening complications such as hepatocellular carcinoma occurrence (HCC), refining the selection of patients with a poorer prognosis in order to intensify anti-viral therapy or HCC screening is of major interest. Among these factors, genetic polymorphisms that could either influence virological response or hepatocarcinogenesis (or both) could be in the future useful markers to improve the selection of HCV-infected patients at higher risk of developing end-stage liver diseases[1].

Chemokines constitute a large family of small (8-10 kDa) cytokines whose effects are mediated by members of a family of 7-transmembrane domain G-pro-

tein coupled receptors[2]. In the liver, chemokines have become increasingly recognized as important mediators of hepatic inflammation[3]. In particular, stromal cell-derived factor-1 (SDF-1)/CXCL12 plays a role in the recruitment and the retention of immune cells in the liver during chronic HCV and hepatitis B virus infection[4]. Lymphocytes infiltrating HCV-infected liver express high levels of the CC chemokine receptor CCR5[5] and the CCR5 ligand, regulated upon activation, normal T cells expressed and secreted (RANTES)/CCL5 may attract naive and activated T cells to the portal and periportal areas[6]. Hepatic stellate cells (HSCs) have been shown to regulate leukocyte trafficking by secreting the CC-chemokine, Monocyte Chemoattractant Protein-1 (MCP-1)/CCL2 and it has been suggested that MCP-1 may have a direct profibrogenic action via HSC chemotaxis[7].

These three major chemokines involved in the pathogenesis of inflammatory liver disease are subject to various genetic polymorphisms[3]. Several studies suggested that chemokine system polymorphisms could be involved in the clinical outcome of HCV-infected patients, either by modulating virological response or by influencing the severity of liver injury. Indeed, significant associations were found between *CCR5-Δ32*, which is a 32-bp deletion in the *CCR5* gene leading to a nonfunctional protein[8], and reduced portal inflammation or milder fibrosis[9], as well as enhanced viral clearance[10]. Furthermore, Promrat *et al* suggested that expression of CCR5 and one of its ligands, RANTES, may be important in the modulation of hepatic inflammation and response to interferon therapy in chronic hepatitis C[11]. This particular *CCR5-Δ32* polymorphism does not appear to be the only chemokine system genetic variant influencing the course of HCV infection; indeed, *MCP-1 (-2518)* allele carriage was found to be associated with higher MCP-1 secretion by hepatic stellate cells and enhanced inflammation and fibrosis[12].

Chemokines have also been shown to be involved in the development of various cancers, mainly breast carcinoma, their physiopathological action including tumour growth, invasion and metastasis[13,14]. Recently, our team showed that SDF-1 stimulates human hepatoma cell growth, migration and invasion, with data obtained in human liver biopsy specimens confirming *in vitro* findings[15]. The homozygous (G to A) mutation at position 801 of the 3'-untranslated region of the *SDF-1* gene, *SDF-1 3'A* has been linked to delayed progression to AIDS in adults with HIV-1 infection[16], but its influence in the course of HCV infection is still unknown.

When taking in account (1) the previously described influence of chemokine system polymorphisms on virological response, liver inflammation and fibrosis in the course of HCV infection and (2) their possible implication in various cancer development and possibly HCC, we hypothesized that these polymorphisms could influence the progression of HCV-related liver injury towards end-stage liver disease. The aim of this work was to study the influence of the most studied polymorphisms *CCR5-Δ32*, *MCP-1 (-2518)* and *SDF-1 3'A* on the prognosis of prospectively followed-up patients with HCV-related cirrhosis, as assessed by the risks of liver-related death and HCC occurrence. As genetic variation affecting the regulatory regions of chemokine genes may modulate their mRNA and protein levels, RANTES, SDF-1α and MCP-1 chemokine serum levels were also determined in these patients.

MATERIALS AND METHODS

Inclusion of patients

The present work is part of a large prospective study, which is ongoing in the department of hepatology of the Jean Verdier hospital, aiming to prospectively assess risk factors involved in HCC development in chronic liver diseases from various etiologies. Among these factors, various genetic polymorphisms have been studied over the past few years including chemokine and chemokine receptor dimorphisms[17-20].

We compiled all new HCV-infected patients who were consecutively referred to our liver unit for diagnosis and treatment between January 1, 1991 and December 31, 2001, and who fulfilled the following inclusion criteria: (1) transparietal or transjugular biopsy-proven cirrhosis; (2) chronic infection by HCV defined by positive serum HCV-RNA; (3) daily alcohol consumption < 20 g; (4) no other cause of liver disease and no infection by the human immunodeficiency virus or hepatitis B virus; (5) no evidence of HCC at the time of inclusion, as judged by negative ultrasonographic findings and serum α-fetoprotein (AFP) less than 50 ng/mL; (6) residence in France; (7) availability of a blood sample to prepare DNA; and (8) acceptance of a regular follow-up for the detection of HCC.

For each patient, the date of inclusion was the date of the first liver biopsy showing cirrhosis. Gender, age, Child-Pugh score, serum alanine aminotransferase (ALT) and aspartate transaminase (AST) levels, platelet count, HCV genotype were recorded at inclusion. The Ethics Committee of our hospital approved the trial.

Follow-up

Patients were prospectively evaluated every 6 mo by physical examination, ultrasonography and AFP measurements. When these investigations suggested possible HCC, computed tomography and/or magnetic resonance imaging and/or a guided liver biopsy were performed according to Barcelona criteria[21].

The two main end-points of the study were the occurrence of HCC, and the occurrence of liver transplantation or death. Follow-up ended at the date of death or liver transplantation, or at the last recorded visit (or information) within the last 6 mo before August 31, 2006, which was set as the final time limit for upgrading the patients' file. The virological response was evaluated in patients who underwent anti-viral treatment. A sustained virological response was defined as the persistence of a negative serum HCV-RNA 6 mo after the end of treatment.

DNA extraction, amplification, CCR5, SDF-1 and MCP-1 genotyping

Genomic DNA was prepared from blood by standard methods[19]. All samples were recoded and blinded. Patients gave written consent for blood sampling and *CCR5*, *SDF-1*, *MCP-1* genotyping. Polymerase chain

Table 1 Characteristics and outcome of patients with HCV-related cirrhosis classified according to MCP1, CCR5 and SDF-1 genotypes

	MCP1 genotype (n = 98)			CCR5 genotype (n = 120)			SDF-1 genotype (n = 120)		
	MCP-1/MCP-1 homozygotes (n = 56) 57.2%	Heterozygotes or homozygotes for *MCP-1 (-2518)* allele (n = 42) 42.8%	P	CCR5/CCR5 homozygotes (n = 106) 88.4%	Heterozygotes or homozygotes for *CCR5Δ32* allele (n = 14) 11.6%	P	SDF-1/SDF-1 homozygotes (n = 65) 54.1%	Heterozygotes or homozygotes for *SDF-1 3'A* allele (n = 55) 45.9%	P
Age (yr)[1,2]	58.7 ± 12.3	58.5 ± 11.8	0.9	58.7 ± 12.1	56.6 ± 13.1	0.7	57.3 ± 11.5	59.8 ± 13.0	0.1
Male gender[1,3]	31 (55.3)	17 (40.4)	0.1	53 (50.0)	8 (57.1)	0.7	35 (53.8)	25 (45.4)	0.4
ALT (× ULN)[1,2]	2.6 ± 1.0	2.9 ± 1.2	0.5	2.8 ± 1.2	2.2 ± 0.6	0.03	2.7 ± 1.8	2.8 ± 1.1	0.7
AST (× ULN)[1,2]	2.1 ± 0.8	2.1 ± 0.7	0.9	2.2 ± 0.8	1.6 ± 0.6	0.008	2.1 ± 0.8	2.2 ± 0.9	0.6
Platelet count (10^3/mm^3)[1,2]	138.3 ± 64.7	142.8 ± 65.0	0.5	138.3 ± 64.7	142.8 ± 65.0	0.5	128.6 ± 60.1	152.9 ± 71.3	0.07
Child Pugh score[1,2]	5.1 ± 0.6	5.1 ± 0.7	0.4	5.2 ± 0.7	5.0 ± 0.2	0.6	5.2 ± 0.8	5.1 ± 0.5	0.2
MCP1 serum levels (pg/mL)[1,2]	448 ± 150	564 ± 168	0.9	-	-	-	-	-	-
RANTES serum levels (pg/mL)[1,2]	-	-	-	28847 ± 18878	29576 ± 15905	0.6	-	-	-
SDF-1 serum levels (pg/mL)[1,2]	-	-	-	-	-	-	2347 ± 690	2201 ± 333	0.7
HCV genotype 1[3]	40 (71.4)	35 (83.3)	0.2	82 (77.3)	13 (92.8)	0.2	49 (75.3)	46 (83.6)	0.3
Anti-viral treatment[3]	32 (57.1)	26 (61.9)	0.6	62 (58.4)	10 (71.4)	0.4	44 (67.9)	28 (52.7)	0.1
Sustained virological[3] Response	14 (25.0)	11 (26.1)	0.9	25 (23.5)	3 (21.4)	0.9	16 (24.6)	12 (21.8)	0.8
HCC development[3]	17 (30.3)	19 (45.2)	0.1	43 (40.5)	4 (28.5)	0.5	26 (40.0)	21 (38.1)	0.7
Death[3]	10 (17.8)	6 (14.2)	0.7	20 (18.8)	3 (21.4)	0.7	11 (16.9)	12 (21.8)	0.4

[1]Parameters recorded at inclusion. [2]mean ± SD. [3]Number (percentage) of patients. ALT: Alanine aminotransferase; AST: Aspartate transaminase; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma occurrence.

reaction amplification of *CCR5* alleles was performed on genomic DNA as previously described[19]. The *SDF-1 3'A* variant (801G to A in the 3'-untranslated region) was detected by electrophoresis on 2.5% agarose gel after PCR amplification and *Msp*I (New England Biolabs, Saint-Quentin-en-Yvelines, France) digestion as previously described[16]. Genotype analysis for *MCP-1 (-2518)* was performed on genomic DNA with PCR with sequence-specific primers followed by restriction fragment length polymorphism analysis as described[22,23]. Briefly, after PCR amplification, PCR products were digested with *Pvu*Ⅱ (recognizes the *MCP-1 -2518* A/G transition) (New England Biolabs).

ELISA assay

All tests were performed using frozen serum collected at fasting and stored at -80℃. SDF-1α, RANTES, MCP-1 serum levels were determined on blood samples collected at inclusion. ELISA assays were performed in patients without evidence of systemic infection at time of blood sample collection as previously described[19].

Statistical analysis

Qualitative variables were compared using the Fischer exact χ^2 test or χ^2 trend test with 1 degree of freedom, while quantitative variables were compared using the non-parametric Wilcoxon test.

The Kaplan-Meier method was used to estimate death and the occurrence of HCC for each parameter noted at enrolment, and the distribution of death and HCC were compared with the Log-rank test[24]. Statistical analysis used the SAS System Package version 8.02 (SAS Institute, Cary, NC).

All reported P values are two-tailed. Associations were first considered statistically significant at a two-tailed α of 0.05.

RESULTS

Characteristics of patients according to the studied genotypes

One hundred and twenty patients were included in this study. All of them had *CCR5* and *SDF-1* genotype assessment and 98 of them had *MCP-1* genotype assessment. Demographic, biological and clinical characteristics according to the *CCR5*, *SDF-1*, and *MCP-1* genotypes are summarized in Table 1. As a very small number of patients were homozygotes for *CCR5-Δ32* allele (n = 0), *SDF-1 3'A* allele (n = 6), *MCP-1 (-2518)* allele (n = 6), we gathered them with heterozygotes in order to compare patients with at least one mutated allele to wild-type homozygotes.

Baseline characteristics estimating the severity of liver disease (prothrombin time, bilirubin levels, albumin, ascites, encephalopathy, Child-Pugh score) were similar among carriers and noncarriers of each genetic marker (Table 1 and data not shown). Demographic data were not different according to allele distributions. Finally, we did not observe any association between the studied chemokine system polymorphisms and the corresponding baseline chemokine serum levels. Thus, there was no association

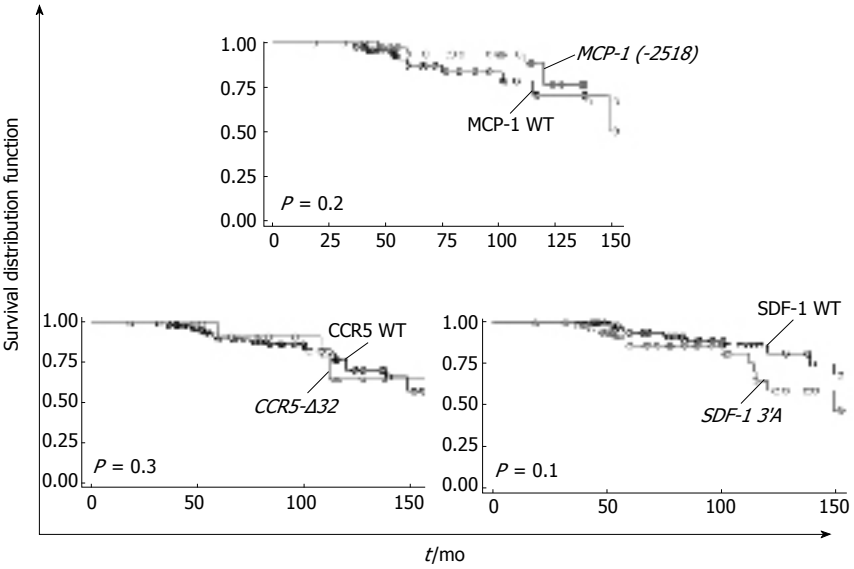


Figure 1 Survival according to *CCR5-Δ32, SDF-1 3'A* and *MCP-1 (-2518)* allele carriage. None of the studied polymorphisms had an influence on survival (at least one mutated allele carriers *vs* wild type homozygotes). *CCR5-Δ32*: Quartile time of survival = 112 mo *vs* 120 mo [RR = 1.02 (95% CI = 0.3-3.4), *P* = 0.3]; *SDF-1 3'A*: Quartile time of survival = 114 mo *vs* 149 mo [RR = 1.9 (95% CI = 0.8-4.4), *P* = 0.1]; *MCP-1 (-2518)*: Quartile time of survival = 139 mo *vs* 115 mo [RR = 0.5 (95% CI = 0.1-1.5), *P* = 0.2].

between the carriage of *CCR5-Δ32* and the serum levels of RANTES, neither between the carriage of *SDF-1 3'A* and SDF-1α serum levels, nor between *MCP-1 (-2518)* allele carriage and MCP-1 serum levels.

Outcome of patients

The incidence of death or transplantation among carriers and noncarriers of each polymorphism is shown in Table 1. Figure 1 displays their prospective influence on these events.

During follow-up, 23/120 (19.1%) patients died or underwent liver transplantation (n = 2). Death was attributable to liver disease in all cases, due to advanced HCC in 19 cases or due to variceal bleeding and/or liver failure in 4 cases. None of the patients included in this study were lost during follow-up. Mean time of follow-up of the cohort was 90.7 ± 43.2 mo.

The incidence of death or transplantation during follow-up was similar among carriers and noncarriers of each genetic marker (Table 1). Using the Kaplan-Meier method, we successively studied the influence of the mutated alleles as risk factors for death/liver transplantation in this cohort. None of the studied polymorphisms had an influence on these events (Figure 1). According to genotypes, quartile time of survival was 112 mo in patients carrying at least one allele *CCR5-Δ32 vs* 120 mo in wild-type homozygotes [RR = 1.0 (95% CI = 0.3-3.4), *P* = 0.3]; 114 mo in patients carrying at least one allele *SDF-1 3'A vs* 149 mo in wild-type homozygotes [RR = 1.9 (95% CI = 0.8-4.4), *P* = 0.1]; 139 mo in patients carrying at least one allele *MCP-1 (-2518) vs* 115 mo in wild-type homozygotes [RR = 0.5 (95% CI = 0.1-1.5), *P* = 0.2].

Seventy-two/120 patients underwent curative antiviral therapy during follow-up. According to genotype distribution, the proportion of treated patients was similar in each group, as well as the infection by HCV genotype 1 which was the most represented in the cohort. None of the studied polymorphisms influenced the sustained virological response (SVR) (Table 1).

Finally, baseline RANTES, SDF-1α or MCP-1 serum levels were not associated with the risk of death/transplantation in our cohort or with the probability of SVR (data not shown).

HCC occurrence

Although inclusion criteria required the absence of detectable HCC at the time of inclusion, 47/120 (39.1%) patients developed HCC during follow-up. Mean time to occurrence of HCC in this cohort was 78.5 ± 40.5 mo.

HCC occurrence during follow-up was not different among carriers and noncarriers of each polymorphism (Table 1). Using the Kaplan-Meier method, we successively studied the influence of the mutated alleles as risk factors for HCC development in this cohort. None of the studied polymorphisms had an influence on this event (Figure 2). According to genotypes, quartile time to HCC occurrence was 72 mo in patients carrying at least one allele *CCR5-Δ32 vs* 68 mo in patients wild-type homozygotes [RR = 0.5 (95% CI = 0.2-1.6), *P* = 0.3]; 72 mo in patients carrying at least one allele *SDF-1 3'A vs* 68 mo in wild-type homozygotes [RR = 1.1 (95% CI = 0.6-2.0), *P* = 0.6]; 67 mo in patients carrying at least one allele *MCP-1 (-2518) vs* 72 mo in patients wild-type homozygotes [RR = 1.1 (95% CI = 0.5-2.1), *P* = 0.8].

Finally, baseline RANTES, SDF-1α or MCP-1 serum levels were not associated with the risk of HCC occurrence in our cohort (data not shown).

DISCUSSION

The results of this study are consistent with a lack of influence of *CCR5-Δ32*, *SDF-1 3'A* and *MCP-1 (-2518)* chemokine system polymorphisms on the prognosis of patients with HCV-related cirrhosis as observed in patients with alcoholic cirrhosis[19]. Our hypothesis, based on the results of several case-control studies displaying an involvement of these genetic variants in the progression of liver injury in HCV-infected patients, was tested on a large cohort of prospectively followed-up patients with a large number of events allowing us to be confident in such a conclusion. The observed discrepancies between the present work and the previously published studies could have several explanations[9,10,12,25].

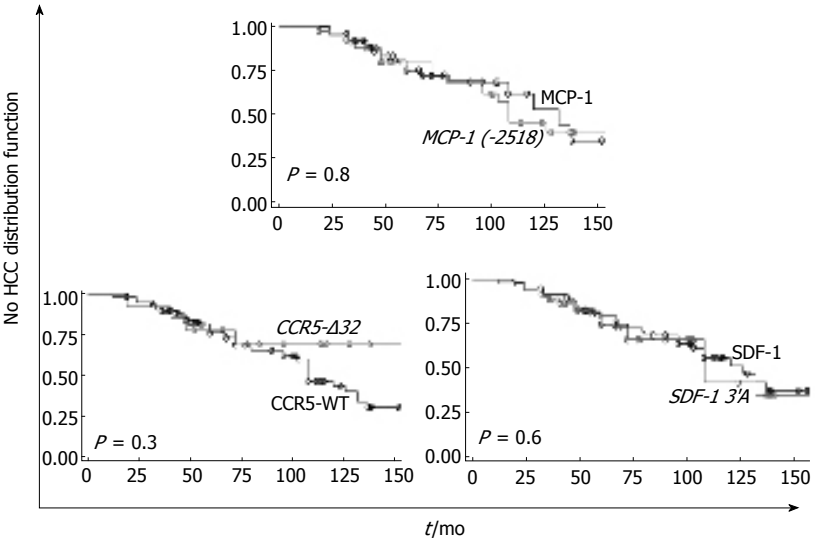


Figure 2 HCC incidence according to *CCR5-Δ32, SDF-1 3'A* and *MCP-1 (-2518)* allele carriage. None of the studied polymorphisms had an influence on the risk of HCC occurrence (at least one mutated allele carriers *vs* wild type homozygotes). *CCR5-Δ32*: Quartile time to HCC occurrence = 72 mo *vs* 68 mo [RR = 0.5 (95% CI = 0.2-1.6), *P* = 0.3]; *SDF-1 3'A*: Quartile time to HCC occurrence = 72 mo *vs* 68 mo, [RR = 1.1 (95% CI = 0.6-2.0), *P* = 0.6]; *MCP-1 (-2518)*: Quartile time to HCC occurrence = 67 mo vs 72 mo [RR = 1.1 (95% CI = 0.5-2.1), *P* = 0.8].

Case-control studies are subject to many methodological biases, including selection bias. Some controversial results have already been reported regarding the involvement of chemokine system polymorphisms in the course of HCV infection. Indeed, Woitas *et al* reported a higher prevalence of the *CCR5-Δ32* allele carriage in HCV-infected patients compared with controls, suggesting that this genetic variant could be associated with a higher risk of developing chronic infection when exposed to the virus[25]. This finding has never been confirmed[9-11,26], raising the issue of selection of specific sub-groups of patients displaying different levels of HCV exposure and the need to confirm such results in independent cohorts. Nevertheless, despite these methodological limitations and controversies[25,27] common to all case-control studies, the influence of chemokine system polymorphisms on HCV-related liver injury relies on solid histological and biological data obtained in human biopsy specimen[9-11].

Our main end-points were the occurrence of liver-related death and HCC in our cohort. These events depend on various and numerous factors, including therapeutic management that can modify the natural course of the disease. As a matter of fact, active preventive procedures aiming to lower life-threatening complications have been carried out in this cohort such as digestive hemorraghe prevention by band ligation or interferon therapy which has been shown to improve survival and lower the risk of HCC incidence in patients with HCV-related cirrhosis[28,29]. As well, as regular HCC screening was carried out in our cohort, patients with small HCC underwent curative treatment, thus modifying their prognosis. Despite the prospective aspect of this study, one could wonder if such therapeutic interventions could induce major changes in the natural course of the disease, thus modifying the importance of chemokine system involvement on the progression of liver injury.

A secondary aim of this work was to assess the influence of the studied polymorphisms on anti-viral therapy. Indeed, several reports showed that CCR5 polymorphisms could be host genetic factors predicting anti-viral treatment success, suggesting that this chemokine receptor could be involved in interferon therapy response[11,30,31]. In the present paper, we did not observe any influence of the studied polymorphisms on anti-viral treatment response.

The functional consequences of the studied polymorphisms are still poorly understood, requiring further *in vitro* and *in vivo* studies aiming to assess if chemokines may be fair serum host factors to explain the inter-individuals differences of evolutions in HCV-infected patients. No association was found between the polymorphisms under study and the baseline serum levels of the corresponding chemokines. Furthermore, these serum levels did not influence the outcome of patients. Nevertheless, the selection of cirrhotic patients is unlikely to be a good choice for the study of the association of polymorphisms with serum levels of a given compound as liver function might affect circulating levels. The assessment of the correlation between chemokine system genetic variants and the corresponding circulating chemokines as well as their possible influence on hepatic injury should be conducted in patients without liver function impairment.

However, these data do not exclude that the chemokine levels in the liver may vary during the course of HCV-related liver disease, as demonstrated by others[32]. Indeed, a statistically significant association between the intrahepatic RANTES expression and the inflammatory activity of chronic hepatitis C was found[32].

It seems reasonable to consider that the progression of liver injury in the course of HCV infection is a continuous pathological process from primo-infection to the development of end-stage liver disease. Nevertheless, mechanisms involved in this progression may not have the same implication before and after the onset of cirrhosis. Thus, the influence of chemokine system could be therefore more critical during the first steps of the infection during which liver inflammation and fibrogenesis are the main physiopathological events. Conversely, their involvement in hepatocarcinogenesis or the progression of liver injury towards liver failure and portal hypertension may not be significant enough (or such events the consequences of too many pathological pathways) to observe an influence of their genetic variants.

In conclusion, the results of this study display a lack of influence of major chemokine system polymorphisms towards liver-related death and HCC occurrence that were previously described as possible host factors influencing the course of HCV infection. This finding highlights the need to assess the prognostic value of such polymorphisms in prospectively followed-up cohorts of patients. If confirmed by other independent cohort studies as previously reported[27], these results suggest that *CCR5-Δ32*, *SDF-1 3'A* and *MCP-1 (-2518)* polymorphisms are not fair candidate genetic variants to select HCV-infected patients at higher risk of developing end-stage liver disease.

COMMENTS

Background

Hepatitis C virus (HCV)-related cirrhosis is a life threatening disease with annual incidences of hepatocellular carcinoma (HCC) and death reaching around 4% and 3% respectively. However, there is wide variability in susceptibility to HCV-related cirrhosis and its outcome such as HCC or death. As epidemiologic factors leading to these complications are well established, the genetic background underlying these differences is still poorly understood and relies on the associations of genetic polymorphisms with such events.

Research frontiers

Polymorphisms in genes encoding for chemokines or chemokine receptors have been associated with the progression of HCV-related liver injury and with various cancer development. However, most of published works are conducted in small-size populations and are case-control studies, thus limiting the reliability of the results observed and the conclusions drawn. Furthermore, their influence on the risks of liver-related death and HCC occurrence in HCV-infected patients is still unknown.

Innovations and breakthroughs

The results of this study are consistent with a lack of influence of chemokine system polymorphisms on the prognosis of patients with HCV-related cirrhosis. This study was carried out in a large cohort of prospectively followed-up patients with a large number of events allowing us to be confident in such a conclusion.

Applications

Exploring new gene variants associated with HCV cirrhosis outcome could be in the future useful markers to improve the selection of HCV-infected patients at higher risk of developing end-stage liver disease.

Terminology

HCC is the most frequent primary liver cancer and the first cause of death in patients with HCV-cirrhosis.

Peer review

This paper shows clearly the lack of any relationship between several genetic markers, the serum level of cytokines and the prognosis of HCV related HCC. Despite their negative results, the study is very well done and described.

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RAPID COMMUNICATION

Double balloon endoscopy associated pancreatitis: A description of six cases

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Abstract

AIM: To perform a single-center analysis of all double balloon endoscopy (DBE) related cases of pancreatitis identified prospectively from a recorded DBE-complication database.

METHODS: From November 2003 until January 2007, 603 DBE procedures were performed on 412 patients, with data on complications recorded in a database. The setting was a tertiary care center offering DBE. DBE was performed from the antegrade or retrograde route. Outcome measurements included age, gender, medication, indication, DBE-endoscope type, insertion depth, procedure duration, findings, interventions, post-procedural abdominal pain, and post-procedural hospitalization.

RESULTS: This is the largest single-center study reporting on post-DBE pancreatitis prospectively. Six patients (1.0%) developed post-DBE pancreatitis, all after antegrade DBE. There was no association with gender, duration of the procedure or type of endoscope. The mean age was 51.9 years (range 25-78). Four patients had severe pancreatitis. Of these, two had inflammatory signs in the body-tail region, one had pancreatitis in the tail region, and the total pancreas was involved in one.

CONCLUSION: The incidence of post-DBE pancreatitis in our series is higher than previously reported. We found no relation with DBE-endoscope type. The inflammatory changes occurred in the body-tail region of the pancreas, suggesting that post-DBE pancreatitis is caused by repetitive mechanical strain on the pancreas.

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Key words: Double balloon endoscopy; Small intestine; Adverse events; Complications; Pancreatitis; Enteroscopy

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INTRODUCTION

Since its introduction in 2001 by Yamamoto[1], double balloon endoscopy (DBE) has revolutionized both diagnostic and therapeutic small bowel endoscopy[2]. This technique allows (intervention) endoscopy of the complete small bowel (often in two sessions) with the possibility of carrying out interventions; e.g. mucosal biopsy, argon plasma coagulation, snare polypectomy, injection therapy, tattooing, foreign body extraction and balloon dilatation. DBE plays an important role in the diagnosis and treatment of obscure gastrointestinal bleeding[3-6], Crohn's disease[7-10], celiac disease[11,12], polyposis syndromes[13] and small bowel tumors. In the series published to date, complications solely related to the diagnostic procedure are rare[14-18]. A recent retrospective multicenter survey indicates a complication rate of 0.8% in diagnostic procedures[16]. In that study, pancreatitis occurred in six of 2362 (0.3%) procedures and contributed to 46% of all complications after diagnostic DBE. Another recent study showed that an asymptomatic rise of serum amylase and lipase levels after antegrade DBE is frequently encountered[19]. Nevertheless, only a few patients develop the clinical picture of post-DBE pancreatitis. The reason for this remains unknown.

This article presents an analysis of all DBE-related cases of pancreatitis identified prospectively from a recorded DBE-complication database of 603 procedures in a single center.

MATERIALS AND METHODS

From November 2003 to January 2007, 603 DBE

Table 1 Patient characteristics

	Gender	Age (yr)	Indication	Scope type	Midazolam (mg)	Fentanyl (mg)	Duration (cm)	Insertion length (cm)	Interventions	Admission (d)	CT inflammation	Peak amylase U/L	Peak lipase U/L
1	M	66	Anemia	P	15	0.100	120	400	Biopsy	7	No CT	334	734
2	M	60	Anemia	P	12.5	0.150	90	250	Biopsy	21	Corpus Tail	412	160
3	F	37	Malabsorption	T	12.5	0.075	90	250	None	2	Tail	619	2037
4	M	54	Anemia	P	10	0.050	80	300	None	11	Total	1395	3660
5	F	23	M.Crohn	P	15	0.150	120	420	None	22	Corpus Tail	1071	369
6	F	44	Small bowel stenosis	T	15	0.050	145	400	Polypectomy	4	No CT	321	120

Reference values: amylase 0-100 U/L, lipase 0-70 U/L.

procedures were performed in an outpatient setting in the Department of Gastroenterology at the VU University Medical Center in Amsterdam, The Netherlands. In our procedures, we used the Fujinon EN-450P5- and the thicker Fujinon EN-450T5 endoscopes. Of the total number of procedures, 311 were performed in men (51.6%) and 292 in women (48.4%) with a mean and median age of 59 and 60 years respectively (range 12-94). Informed consent was obtained from all patients in accordance with the institutional review board guidelines. Data recorded in the complication database included age, gender, co-morbidity, surgical history, medication, indication for DBE, depth of insertion as described by May[20], procedure duration, findings, interventions performed, post-procedural abdominal pain, and post-procedural hospitalization. The patients received a combination of midazolam and fentanyl intended for conscious sedation.

The complication database was reviewed. All patients with post-DBE pancreatitis were identified and their charts were reviewed for additional clinical information including severity of pancreatitis, length of hospitalization, peak serum CRP, peak serum amylase, peak serum lipase, abdominal imaging performed, surgery performed and mortality.

According to the literature on post-endoscopic retrograde cholangiopancreatography pancreatitis (ERCP; American Society of Gastrointestinal Endoscopy (ASGE) guidelines), post-DBE pancreatitis was defined as newly developed abdominal pain after a DBE-procedure, requiring at least 2 d of unplanned hospitalization, with a serum amylase $\geqslant$ 3 as the upper limit 24 h after the procedure[21]. According to these guidelines, the severity of the disease was classified as follows: mild pancreatitis requiring 2-3 d of hospitalization; moderate pancreatitis requiring 4-10 d of hospitalization; severe pancreatitis requiring 10 or more days of hospitalization, and/or the occurrence of pseudocysts and/or the need for surgery.

Statistical analysis

The different parameters were compared with t-tests. Results were considered significant if P values were < 0.05.

RESULTS

In a total of 603 DBE procedures, six patients (1%) developed post-DBE pancreatitis. Case summaries are presented in Table 1. Fifty percent of the patients ($n = 3$) was male. The mean age was 47.3 years (range 23-66). DBE was performed for the following indications: iron deficiency anemia ($n = 3$), Crohn's disease ($n = 1$), malabsorption ($n = 1$) and small bowel stenosis after bowel surgery ($n = 1$). The route of the procedure was oral in all 6 patients. In 67% ($n = 4$) the P-type endoscope (Fujinon EN-450P5) was used. The mean duration of the procedures was 108 min (80-148), the mean depth of insertion was 337 cm (250-420). In one patient, a polypectomy was performed.

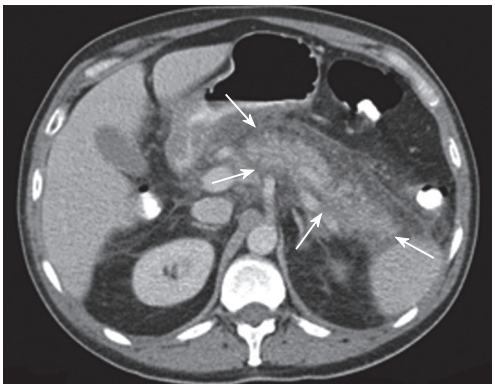


Figure 1 A CT image of a 23-year old female, who developed post-DBE pancreatitis (case 5), showing an inflamed body-tail region, with edema and fatty infiltration (arrows).

All six patients developed clinical signs of pancreatitis within 6 h after the procedure. A scoring system to record the intensity of the pain in the patients was not maintained. The mean duration of admission was 11.2 d (2-22). The mean peak amylase and lipase levels were respectively 692 U/L (321-1395) and 1180 U/L (120-3660). Two patients developed moderately severe pancreatitis (case 1 and 6), and four had severe pancreatitis (case 2-5). In four patients, CT-scan was performed. Two of these patients showed inflammatory signs of the body-tail region of the pancreas with edema and fatty infiltration. One patient had pancreatitis in the tail of the pancreas and developed a pseudocyst during admission (case 3). In one patient, the whole pancreas was involved (Figures 1 and 2). Two patients had moderate clinical and laboratory signs of disease and could be discharged within a few days of admission. Therefore, CT imaging was not performed. One patient underwent laparotomy because postprocedural abdominal pain was suspected to be caused by perforation.

Table 2 Procedural characteristics

	Pancreatitis (n = 6)		No Pancreatitis (n = 597)		
Age (yr)	47.3	(23-66)	59.3	(12-94)	
Gender					
Male	3	(50%)	301	51.60%	
Female	3	(50%)	292	48.40%	
Route					
Oral	6	100%	435	73%	
Anal	0		162	26%	
Endoscope					
P-type	4	67%			
T-type	2	33%			
Duration (min)	108	(80-148)	105	(30-240)	
Insertion depth (cm)	337	(250-420)	270	(25-640)	P = 0.09
Midazolam (mg)	13.3	(10-15)	10	(2.5-25)	
Fentanyl (mg)	0.1	(0.05-0.15)	0.075	(0.025-0.2)	

However, during surgery, pancreatitis was diagnosed and no perforation was present. The other five patients were treated conservatively. There was no mortality in our series.

Table 2 shows clinical and procedural characteristics of the patients that developed acute pancreatitis in comparison with the patients that did not develop pancreatitis. All patients who developed post-DBE pancreatitis had undergone antegrade DBE. Depth of insertion was higher in the patients developing pancreatitis than in those who did not, although this difference did not reach statistical significance.

DISCUSSION

In our series, post-DBE pancreatitis developed after six of 603 procedures (1%). This rate is higher than previously reported in the literature. A study by Heine *et al*, in which our center participated, identified 3 cases of post-DBE pancreatitis in a total of 275 procedures[22]. Two of those patients are also described in the present study. In a prospective multicenter study by Ell and colleagues, 100 patients were included and no pancreatitis occurred[23]. In a retrospective analysis of 378 DBE procedures by Zhong *et al* 2 patients suffered an unspecified rise of serum amylase in conjunction with abdominal pain, which resolved after conservative measures[24]. Whether these patients required hospitalization is not stated. The multicenter questionnaire survey by Mensink *et al* identified 6 cases of pancreatitis in 1728 diagnostic procedures (0.3%) and 1 case of pancreatitis after DBE-assisted ERCP in a patient with a Roux-en-Y reconstruction[16]. Several possibilities should be considered when trying to explain the difference between post-DBE pancreatitis rates in the present study and the previously published studies. First of all, the method of insertion and withdrawal might be a factor in the origin of post-DBE pancreatitis. However, we are not aware of any differences in technique between our center and other centers, while there is close contact and exchange of knowledge with many DBE-performing centers inside and outside The Netherlands. The fact that trained fellows under close supervision regularly perform DBE in our center, and that pancreatitis could be a consequence of relatively inexperienced endoscopists, is also an unlikely explanation since this would most certainly have been reflected in total procedure time, which was almost the same in patients developing pancreatitis and those who did not. Additionally, post-DBE pancreatitis did not occur more frequently after procedures performed by fellows. A third possible explanation could be an underestimation of post-DBE pancreatitis in previously published studies, as is evident in the study by Zhong, which reports two patients with abdominal pain and increased serum amylases without labeling these cases as pancreatitis[24]. Especially, retrospective questionnaire based surveys might be at risk from report or inclusion bias. Differences in definitions of post-procedural complications offer a likely explanation for the difference in reported post-DBE pancreatitis. Since we conducted a review of a recorded database prospectively, using predefined definitions of pancreatitis and its severity, the chance of report or inclusion bias seems lessened.



Figure 2 A transversal CT-image of a 54-year old male, who developed post-DBE pancreatitis (case 4), showing edema and fatty infiltration around the pancreas.

The pathogenesis of post-DBE pancreatitis is not known. Honda *et al* found asymptomatic hyperamylasemia in 5 of 13 patients after antegrade DBE. One of 13 patients developed pancreatitis[25]. Several mechanisms could be responsible for the development of hyperamylasemia or pancreatitis. First, the repetitive mechanical strain on the pancreas, by inserting the endoscope/overtube, by inflated balloons or by stretching movements, could induce pancreatitis, especially by traction at the ligament of Treitz. This hypothesis is supported by the finding that in most cases in which an early CT scan was performed, pancreatitis was located in the tail or body/tail region of the pancreas. As in the study by Mensink *et al*, no significant association between the use of the T-endoscope and the occurrence of pancreatitis was present. This strain-hypothesis is also suggested by our finding that insertion depth (and therefore the number of advancing and stretching movements of both endoscope and overtube) seems higher in the pancreatitis group (P = 0.9, Table 2). A second explanation could be duodenal hypertension due to air trapping between the overtube balloon and the pylorus, which in theory could interfere with pancreatic drainage. In fact, a closed duodenal loop is an established animal model for acute pancreatitis[26-29]. As far as we know, data that could prove this theory do not exist and our finding that procedure time (and therefore the time the supposed duodenal hypertension would have existed) was not different between the groups is not supportive of this theory. Impaired pancreatic drainage

due to temporary obstruction of the papilla could not have resulted in pancreatitis in our series, since we inflate the balloons after Treitz's ligament has been passed.

To our knowledge no cases of pancreatitis have been reported after retrograde DBE-procedures, therefore it is highly unlikely that medications used for sedation could have played an important role. We also noted that all the females that developed post-DBE pancreatitis were premenopausal. However, this series is too small to identify patient-related risk factors. It is therefore unknown whether patient characteristics associated with post-ERCP pancreatitis (like sphincter of Oddi dysfunction, young age or female gender) are also associated with the occurrence of post-DBE pancreatitis[30]. Larger, prospective multicenter studies are needed to investigate operator-dependent factors and to identify additional patient-related risk factors that could explain why some patients develop only transient hyperamylasemia and others severe pancreatitis. Such studies should include early pancreatic imaging. Anatomical and imaging studies on the effect of DBE on the position of the pancreas and proximal duodenum are necessary to investigate the validity of the pancreatic strain and the duodenal hypertension hypotheses.

Although post-DBE pancreatitis is relatively uncommon, it is the most important complication of antegrade, diagnostic DBE. It is of major importance to consider pancreatitis as a cause of post procedural abdominal pain, to avoid unnecessary surgical exploration as has been the case in one of our patients. Abdominal pain after DBE warrants additional investigation. Since hyperamylasemia is quite common after DBE and not discriminative for DBE pancreatitis, the diagnosis should be based on clinical findings and or radiological imaging techniques.

COMMENTS

Background

Double balloon endoscopy (DBE) was introduced by Yamamoto in 2001. This endoscopic technique allows (intervention) endoscopy of the small bowel. Post DBE pancreatitis is a well known complication after antegrade DBE.

Research frontiers

This article describes the cases of post DBE pancreatitis in the largest single center series of DBE procedures to date.

Related publications

Honda *et al* showed that an asymptomatic rise of serum amylase and lipase levels after antegrade DBE occurs frequently. Nevertheless, previous studies indicate that post DBE pancreatitis occurs seldom.

Innovations and breakthroughs

The pathophysiology behind the occurrence of post DBE pancreatitis largely remains unknown. Only a few reports on this subject have been published. Studies to determine the pathogenesis are currently performed in our center.

Applications

The investigation post DBE pancreatitis will help us to determine the pathogenesis of this complication, to define high risk groups, and thus to prevent post DBE pancreatitis.

Terminology

DBE is a endoscopic technique that allows access to the complete small intestine for diagnostic and therapeutics procedures.

Peer review

It is a good manuscript reporting six cases of DBE-associated pancreatitis.

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RAPID COMMUNICATION

Outcome of patients with acute, necrotizing pancreatitis requiring drainage-does drainage size matter?

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Abstract

AIM: To assess the outcome of patients with acute necrotizing pancreatitis treated by percutaneous drainage with special focus on the influence of drainage size and number.

METHODS: We performed a retrospective analysis of 80 patients with acute pancreatitis requiring percutaneous drainage therapy for infected necroses. Endpoints were mortality and length of hospital stay. The influence of drainage characteristics such as the median drainage size, the largest drainage size per patient and the total drainage plane per patient on patient outcome was evaluated.

RESULTS: Total hospital survival was 66%. Thirty-four patients out of all 80 patients (43%) survived acute necrotizing pancreatitis with percutaneous drainage therapy only. Eighteen patients out of all 80 patients needed additional percutaneous necrosectomy (23%). Ten out of these patients required surgical necrosectomy in addition, 6 patients received open necrosectomy without prior percutaneous necrosectomy. Elective surgery was performed in 3 patients receiving cholecystectomy and one patient receiving resection of the parathyroid gland. The number of drainages ranged from one to fourteen per patient. The drainage diameter ranged from 8 French catheters to 24 French catheters. The median drainage size as well as the largest drainage size used per patient and the total drainage area used per patient did not show statistically significant influence on mortality.

CONCLUSION: Percutaneous drainage therapy is an effective tool for treatment of necrotizing pancreatitis. Large bore drainages did not prove to be more effective in controlling the septic focus.

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Key words: Acute necrotizing pancreatitis; Percutaneous drainage; Drainage size; Interventional radiology; Percutaneous necrosectomy

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INTRODUCTION

The course of acute pancreatitis ranges from a mild transitory edematous to a severe necrotizing form. Necrotizing pancreatitis occurs in about 20% of all patients suffering from acute pancreatitis[1]. If infection of the necrotic tissue occurs mortality rates of up to 50% are reported with sepsis and multiorgan failure as most frequent causes[2-5]. It is generally accepted that in infected necrotizing pancreatitis the infected non-vital solid tissue has to be removed in order to control the sepsis. The standard treatment has traditionally been surgery[5,6]. By using modern surgical techniques like open packing, repeated laparatomies, closed packing or closed continuous lavage mortality rates could be decreased to 20%-40%[7-12]. However, these techniques are associated with a considerable surgical trauma which often causes escalation of multiorgan failure and sepsis[7,13]. Moreover, total anaesthesia is mandatory. Thus, in the last decade minimal invasive treatment regimes and in particular percutaneous drainage therapy were included in the management of infected necrotizing pancreatitis.

Ultrasound (US) or computed tomography (CT) guided placement of drainages is reported to be effective in up to 90% for drainage of fluid collections or abscesses with purely liquid content[14]. However, the success rates

for drainage of contained fluid collections in necrotizing pancreatitis are less convincing ranging between 26% and 50%[15,16], with a subsequent frequent need for further surgery. The presumed reason is that infected fluid collections/abscesses in necrotizing pancreatitis are often not completely liquid but contain solid debris. The value of drainage therapy for removal of solid debris is equivocal. Usage of large bore catheters and placement of several drainages is generally considered to be more effective. This hypothesis, however, has not yet been evaluated systematically.

The aim of this retrospective study was to evaluate if patient outcome in infected necrotizing pancreatitis correlates with the number and size of percutaneously placed drainages.

MATERIALS AND METHODS

Patient recruitment and review of data

This is a retrospective study performed at a university hospital. By searching the radiological, surgical and internal medicine databases all patients with the diagnosis acute pancreatitis treated with drainages between 1992 and 2004 were identified. Demographic (age, sex), clinical (length of primary hospital stay, eventual second hospital stay, admittance to intensive care unit, etiology of pancreatitis, diagnostic and conservative therapeutic approaches), laboratory data and characteristics of surgical procedures were collected by reviewing patient charts and surgical reports.

Number of CTs performed per patient and characteristics of percutaneous interventional therapy were collected by reviewing all radiological reports. In particular, number of drainages per patient, date of drainage placement, size of each drainage in French, largest drainage per patient according to French number, estimate of draining lumen of each drainage (by calculating the axial plane of each drainage), and length of drainage placement in days were determined. Moreover, the cumulative draining area (by adding the draining lumina of all drainages per patient), the average draining area (by calculating the average of the draining lumina of all drainages per patient), the cumulative diameter (by adding the diameter according to French number of all drainages per patient), and the average diameter (by calculating the average of the diameter according to French number of all drainages per patient) were calculated.

All drainages had been placed under CT guidance using Seldinger technique. The number and the size of catheters were determined individually for each patient by the intensive care unit (ICU) team and the interventional radiologist. Clinical considerations and the size and location of necroses as well as the suspected amount of fluid and debris (based on CT findings) guided this decision.

For patients with ongoing sepsis and solid tissue within the fluid collections, active necrosectomy was performed. This technique has been described in detail elsewhere[16-18]. Briefly, under local anaesthesia the drainage was replaced by a 24F-30F peel away sheath. Under fluoroscopic and/or endoscopic guidance the solid tissue was fragmented and extracted through the sheath. For this, snare catheters, dormia baskets, forceps and brisk flushing/aspiration were applied.

For patients receiving intensive care treatment, the APACHE Ⅱ score, SAPS Ⅱ, and Ranson score were determined by reviewing the laboratory data and the ICU flow charts.

The Balthazar score and computed tomography severity index (CTSI) were determined by retrospectively reviewing the CT examination at the time of first drainage placement[19]. Radiological analysis was done by a board certified radiologist with 6 years of experience.

Endpoints for patient outcome during hospital stay were defined as patient death and length of hospital and/or ICU stay. All patients were observed until d 250 or censored at the last day of observation.

A telephone interview was performed to investigate long term outcome parameters. The questionnaire included life quality parameters such as ongoing symptoms of pancreatitis after hospital discharge (abdominal pain, diarrhea, intolerance of fat, weight loss), development of exocrine or endocrine dysfunctions, repeated hospital stays and need for further surgery.

Statistical analysis

Parameters assessing drainage characteristics and clinical scores (for ICU patients) were correlated with mortality using logistic regression. Drainage characteristics and clinical scores (for ICU patients) were correlated with length of hospital stay and ICU stay using a Cox proportional hazard model in a univariate model. Variables with a significant influence on the hazard ratio were then included in a multivariate model. The statistical analysis was made with the SPSS 12.0 statistical software (SPSS inc., Chicago, IL). Values are given as total numbers, median with range or as percentages where necessary. A P-value $\leqslant 0.05$ was considered statistically significant.

RESULTS

Patient population

Eighty patients fulfilled the inclusion criteria and were included in the study. The demographic, clinical, laboratory and interventional/surgical data are shown in Table 1.

Clinical and laboratory data

All 80 patients received antibiotic treatment over a median of 44 d with a range from 2 d to 205 d. Antimycotic treatment was established in 43 patients (54%). The diagnosis of infection of the necrotic tissue was based on the examination of aspirated material with Gram´s stain, culture or both. Fine needle aspiration was not routinely performed. Thus, the material which was obtained during the placement of the first drainage was evaluated. Fifty-two patients (65%) showed positive microbiological results. The other 35% might have shown false negative results due to prior initiation of antibiotic therapy.

Intensive care treatment was necessary in 65 out of 80 patients (81%); nine patients required a second ICU

Table 1 Patient characteristics

Patient, *n* = 80	*n*/median (%/range)
Demographics	
Age	57 (17-79)
Sex, female	26 (32.5)
Days of hospitalisation	51 (3-241)
Required second hospitalisation	5 (6.3)
Aetiology	
Biliary	26 (32.5)
Alcoholic	32 (40.0)
Post-ERCP	3 (3.8)
Toxic	1 (1.3)
Hyperlipidemia	1 (1.3)
Hyperparathyroidism	1 (1.3)
Posttraumatic	1 (1.3)
Idiopathic/unknown	15 (18.8)
Ranson score	2 (0-4)
Diagnostic procedures	
Ultrasound	74 (92.5)
ERCP	32 (40.0)
CT	80 (100)
Balthazar score	4 (3-4)
CTSI	6 (4-10)
Inflammatory markers	
Leucocyte count d 1 of our hospital admission (/nL)	13 (3.5-55.0)
Leucocyte count on day of first drainage placement (/nL)	14.4 (3.5-46.6)
C-reactive protein day 1 (mg/L) of our hospital admission	208 (10-477)
C-reactive protein on day of first drainage placement (mg/L)	191 (20-477)
Therapy	
Surgical	20 (25.0)
Minimal-invasive	
Number of drainages, range	2 (1-14)
Active necrosectomy	18 (22.5)
Intensive care treatment	65 (81.3)
length of ICU stay, range	22 (2-104)
Required second ICU stays	9 (11.3)
APACHE Ⅱ, range	18 (1-38)
SAPS Ⅱ, range	61 (15-94)
Non-invasive positive pressure ventilation	2 (2.5)
Mechanical ventilation	50 (62.5)
Renal replacement therapy	23 (28.8)
Plasma exchange therapy	7 (8.8)
Mortality	27 (33.8)
Cause of Death	
Septic multi-organ failure	24 (88.9)
Pulmonary embolism	1 (3.7)
Intracerebral bleeding	1 (3.7)
Due to polytrauma	1 (3.7)

ERCP: Endoscopic Retrograde Cholangiopancreatography; CT: Computed Tomography; CTSI: Computed Tomography Severity Index; ICU: Intensive Care Unit; APACHE: Acute Physiology and Chronic Health Evaluation; SAPS: Simplified Acute Physiology Scale.

stay later on. Mechanical ventilation was necessary in 50 patients (63%), renal failure with the need of renal replacement therapy occurred in 23 patients (29%).

Radiological data

Each patient received a median of six CTs ranging from 1 to 23, which were all contrast enhanced. The CTSI of all 80 patients was in median 6 ranging from 4 to 10, the Balthazar score was in median 4, ranging from 3 to 4.

Necrosis of the pancreas was diagnosed in 73 patients (91%) on the basis of contrast enhanced CT findings. The location of necrosis was only in the head of the pancreas in 13 patients, in the head and the body in five patients, in the head and the tail in four patients, in the body in three patients, in the body and tail in 10 patients, only in the tail in six patients and involving the whole pancreas in 32 patients. Seven patients did not show necrosis of the pancreas itself, however, extensive peripancreatic fluid collections with presumed necrosis of the peripancreatic fat was present.

Characteristics of drainage therapy

Different catheter types were used (Thal-Quick, Cook, Europe; van Sonnenberg, Peter Pflugbeil, Zorneding, Germany; APD and Flexima, Boston Scientific, Natick, USA). Up to 14 catheters per patient were placed, in median two catheters per patient. Percutaneous catheter drainage was performed on median d 3.5 after admission (range 1 d to 40 d), but with 71 patients being transferred from peripheral hospitals and median hospitalisation of 7 d before (complete data of prior hospital stays was available in 54 out of 71 patients). Drainages remained in situ for a median of 36.5 d (range 1 d to 260 d). The drainage size ranged from 8 French to 24 French catheters. The median drainage plane was 16.9 mm^2, the median cumulative drainage plane was 37.0 mm^2 per patient. The median diameter was 14.0 mm, the median cumulative diameter was 30.0 mm per patient.

No acute complication (like bleeding, bowel perforation, accidental puncture of liver, spleen or kidney) related to catheter placement occurred. However, in 23 patients (29%) the development of fistulas in the pathway was observed later on.

Catheters were exchanged in median two times per placed catheter (range from 1 to 9). Catheter exchange was either done because of drainage blockage or to insert a larger drainage for presumed better drainage of necrotic tissue.

In 18 of 80 patients (23%) an active percutaneous necrosectomy was performed. Ten patients out of these required surgical necrosectomy later on. Six patients underwent surgical necrosectomy without prior percutaneous necrosectomy. Elective surgery was performed in 4 patients (cholecystectomy in three patients and adenomectomy of the parathyroid gland for primary hyperparathyreoidism in 1 patient) during the same hospital stay (Figure 1).

Endpoints

Length of hospital stay was in median 51 d with a range from 3 d to 241 d. A second hospital stay was necessary in 5 patients (6%). The length of stay in the ICU ranged from 2 d to 104 d, in median 22 d. The overall mortality was 34% (27 of 80 patients).

The correlations of the radiological and clinical scores (CTSI, APACHE Ⅱ, SAPS Ⅱ), clinical treatment modalities (invasive ventilation and renal replacement therapy) as well as interventional treatment modalities (number of drainages, median drainage size, largest drainage size and total drainage lumen) with mortality

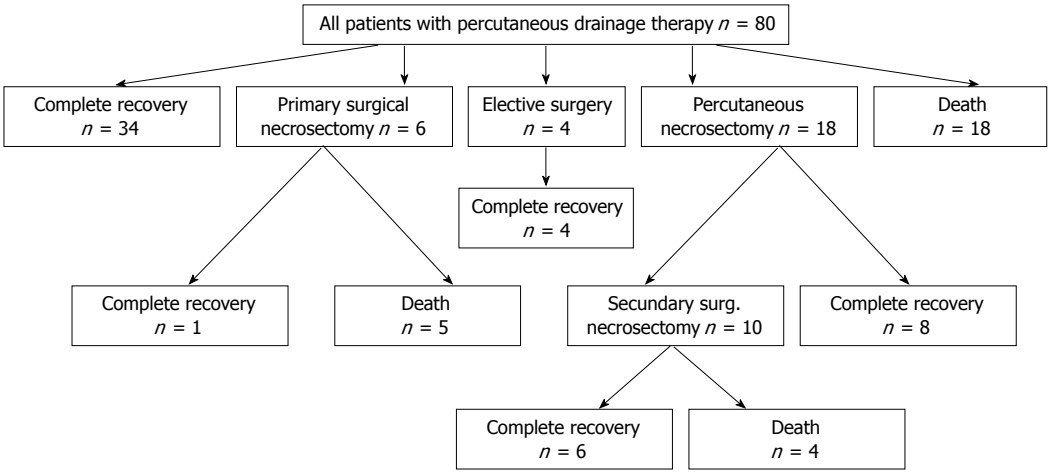


Figure 1 Therapeutic procedures.

Table 2 Cox proportional hazard model for overall mortality using uni- and multivariate analysis

Variable	Mono-HR	*P*	Multi-HR	*P*
Age	0.985	0.098	-	
Sex	0.926	0.793	-	
CTSI	0.933	0.281	0.982	0.819
Apache Ⅱ Score	0.961	0.016	0.989	0.716
SAPS Ⅱ Score	0.976	0.002	1.004	0.809
Clinical treatment modalities				
Invasive ventilation	0.175	0.000	5.618	0.001
Renal replacement therapy	0.329	0.005	2.347	0.046
Interventional treatment modalities				
Number of drainages	0.892	0.077	-	
Median drainage size	0.989	0.830	-	
Largest drainage size	0.954	0.307	-	
Total drainage plane	0.994	0.052	0.993	0.084

Mono-HR: Monovariate hazard ratio; Multi-HR: Multivariate hazard ratio; APACHE: Acute Physiology and Chronic Health Evaluation; SAPS: Simplified Acute Physiology Scale; CTSI: Computed Tomography Severity Index.

Table 3 Outcome parameters of patients after treatment with percutaneous drainage applied 1-12 years after hospital discharge by a telephone investigation

Outcome parameters from 29 patients	*n*	%
Life quality parameters		
Abdominal Pain	5	17.2
Diarrhea	7	24.1
Intolerance of fat	4	13.8
Weight loss	7	24.1
Exocrine pancreatic dysfunction (with substitution of pancreatic enzymes)	7	24.1
Endocrine pancreatic dysfunction	10	34.5
Repeated development of pancreatitis	3	10.3
Further hospitalisations	8	27.6
Surgery after discharge	7	24.1
Death after discharge	4	13.8

are given in a cox proportional hazard model for overall mortality using uni- and multivariate analysis (Table 2). Significant risk factors for mortality in univariate analysis were the need for mechanical ventilation, renal replacement therapy, the APACHE Ⅱ score and the SAPS Ⅱ score. The total drainage plane showed borderline significance with a *P*-value of 0.052. The median drainage size, the largest drainage size used per patient and the CTSI were not significantly correlated with mortality. In the multivariate model the CTSI was as the only not significant variable included adjusting for the extent of necrosis. Renal replacement therapy and the need for ventilation support were the only parameters which remained significant in the multivariate model.

Long-term outcome

In 29 out of the 53 surviving patients (55%) long-term outcome parameters could be collected. Data are presented in Table 3. Hereby endocrine pancreatic dysfunction is the most frequent factor affecting life quality (35%), followed by diarrhea, weight loss and exocrine pancreatic dysfunction with the need of enzyme-substitution in 24%; 10% reported on repeated pancreatitis episodes; 28% on further hospital stays for related symptoms.

DISCUSSION

Percutaneous drainage is the therapy of choice for treating fluid collections. The success rates for drainage of fluid collections, pseudocysts or simple abscesses in variable sites are reported to be around 90%[20-24]. In contrast, reports on percutaneous drainage in patients with acute necrotizing pancreatitis were overall disappointing showing drainage therapy to be often inefficient with subsequent need for open surgery[15,25]. The presumed reason for this discrepancy is that pancreatic/peripancreatic necroses and pancreatic abscesses contain solid, avital debris. However, some studies reported significantly better success rates treating necrotizing pancreatitis by drainage therapy only[15,26,27]. A common feature of these studies was that large bore catheters (up to 28F) were used. The rationale for this strategy was the hypothesis that large bore catheters might be more effective for mobilizing solid tissue. However, the drawback of using large catheters is the more traumatic approach with the risk of bleeding and organ injury. In the present study, we evaluated the effect of drainage characteristics on the outcome of patients with necrotizing pancreatitis.

The 80 patients included in this study all suffered from acute, necrotizing pancreatitis. The diagnosis was based on the results of contrast-enhanced CT as well as clinical and laboratory findings. Seventy-three patients presented with necrosis of the pancreatic parenchyma itself, 7 patients demonstrated presumed necrosis of peripancreatic tissue. No patients with pseudocysts were included. Fifty-two patients (65%) had microbiologically proven infection of the necrotic tissue. Although for 28 patients (35%) culture was negative, interventional therapy was initiated because the clinical, laboratory and radiological findings strongly suggested infection.

The gold standard for the treatment of infected, necrotizing pancreatitis still is open surgery[28]. However, minimal-invasive interventional approaches continue to gain importance. In the present study, 43% of patients were cured by percutaneous drainage and conservative treatment only. When including those patients who additionally were treated with active, percutaneous necrosectomy without the need for open surgery the cure rate increased to 53%. Six surviving patients (8%) required surgical necrosectomy because percutaneous therapy failed in controlling sepsis. The mortality rate in this study was 34% which is within the expected range of 20%-40%[10-12] reported in the literature. Considering that the study population represented a seriously ill subset of patients as evidenced by high clinical and radiological scores, a mortality rate of 34% is an acceptable outcome.

The time of placement of the first drainage after hospital admission with a median of 3.5 d seems quite short because infection of necroses occur usually within 7 d to 10 d after the onset of symptoms[2,28]. However, it has to be considered, that 71 out of 80 patients (89%) were transferred from other hospitals with median hospitals stays of 7 d before the transfer to the university hospital. Due to the retrospective nature of this study we were able to assess the time of hospital admission, which naturally was not always identical with the time of symptom onset.

As opposed to the APACHE Ⅱ and SAPS Ⅱ scores the CTSI score did not show a good correlation with patient outcome in our study. This is discrepant to results published by Balthazar *et al* who found the mortality and morbidity to be closely correlated to the CTSI[19]. However, Balthazar *et al* studied a patient population covering the whole range of possible CTSI values whereas our population was seriously ill with CTSI values of at least 4. Presumably, the CTSI is not a good parameter to predict outcome in this subset of patients.

No significant correlation between drainage size/number and outcome parameters was found in this study. Rather than abolishing the concept of using large bore drainages we think that our results reflect the fact that the outcome of patients suffering from infected, necrotizing pancreatitis is influenced by a multitude of factors. 81% of the patients in this study required ICU admission with the need for mechanical ventilation and renal replacement therapy in many cases. It seems to be difficult to isolate one single parameter which might modify overall prognosis in such a complex study group. An aggravating factor was the retrospective nature of this study. Different lavage schemes and various types of drainages were used over the years and the size of drainages used is influenced by a multitude of factors. Especially the latter fact is important since the inner diameter of the adapter and of the drainage itself is different for different companies despite the identical outer diameter given in French. Moreover, the length of the drainage is inversely correlated with the flow. A prospective study seems to be necessary to evaluate the impact of drainage characteristics. However, it is questionable if such a study is feasible because large study groups are necessary to provide adequate power.

In conclusion, percutaneous drainage and minimal invasive necrosectomy are efficient tools for treatment of necrotizing pancreatitis. Large bore drainages did not prove to be more effective in controlling the septic focus. However, it seems to be difficult to determine a single prognostic parameter in these multimorbid patients.

COMMENTS

Background

Percutaneous, interventional treatment modalities are safe and efficient tools in the therapy of patients with necrotizing pancreatitis. Special therapy details, like the optimal drainage size, have not yet been evaluated.

Research frontiers

Whereas surgical debridement is often associated with a considerable trauma and may cause escalation of multiorgan failure and sepsis, minimal invasive methods are a safe and effective tool to bridge time to surgery or avoid it at all.

Innovations and breakthroughs

In this article we analyze if drainage size affects outcome in 80 patients with necrotizing pancreatitis.

Applications

Large bore drainages (≥ 16F) did not prove to be more effective in controlling the septic focus than smaller ones (< 16F).

Terminology

Interventional methods in our article include percutaneous, radiologically assisted drainage therapy.

Peer review

This is a well written manuscript. Publication is recommended.

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RAPID COMMUNICATION

Peripheral corticotropin releasing hormone mediates post-inflammatory visceral hypersensitivity in rats

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Abstract

AIM: To investigate whether peripheral corticotropin releasing hormone (CRH), which is up-regulated in intestinal inflammation, mediates the post-inflammatory visceral hypersensitivity in a rat model of colitis.

METHODS: We measured mucosal myeloperoxidase (MPO) activity as a marker of inflammation, plasma CRH level, and abdominal withdrawal reflex (AWR) to colorectal distension as a visceral nociceptive response at 2, 7 and 14 d after the induction of colitis with 4% acetic acid.

RESULTS: Colonic inflammation, quantified by MPO activity, significantly increased on d 2 and subsided thereafter, which indicated a resolution of inflammation within 7 d. On the contrary, plasma CRH level and AWR score were increased on d 2, remained high on d 7, and returned to control level on d 14. Intraperitoneal injection of a CRH antagonist, astressin (30 μg/kg), significantly attenuated the post-inflammatory visceral hypersensitivity on d 7. Furthermore, intraperitoneal administration of CRH (3 and 10 μg/kg) mimicked the post-inflammatory visceral hypersensitivity in naive rats.

CONCLUSION: These results suggest that increased peripheral CRH mediates the enhanced visceral nociception in rats recovered from experimental colitis.

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Key words: Corticotropin releasing hormone; Visceral hypersensitivity; Colitis

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INTRODUCTION

Visceral hypersensitivity, which refers to a lowered threshold for visceral pain/discomfort elicited by intraluminal mechanical/chemical stimuli, is a hallmark symptom of irritable bowel syndrome (IBS)[1,2]. IBS is a common functional gastrointestinal disorder that has a great impact on quality of life. It is thought that previous episodes of intestinal inflammation predispose to IBS as intestinal inflammation induces profound changes that persist even after the inflammation subsides[3]. Indeed, patients with ulcerative colitis in remission often express IBS-like symptoms[4], and animals that have recovered from experimental enteritis show visceral hypersensitivity to luminal distension[5,6]. Nevertheless, it has still not been completely elucidated what causes this IBS-like, post-inflammatory visceral hypersensitivity.

Increasing evidence suggests that peripheral corticotropin releasing hormone (CRH) plays an important role in intestinal inflammation. Kawahito *et al*[7] have reported a significant increase in the number of CRH immunoreactive macrophages and enterochromaffin cells in the colonic mucosa of patients with inflammatory bowel disease. In a rat model of chronic granulomatous enterocolitis, both mRNA and protein expression of CRH was found to be increased in cecal inflammatory cells, mesenchymal cells, and myenteric plexi[8]. More recently, an increase in CRH and its receptors was reported in a mouse ileitis model injected with *Clostridium difficile* toxin A[9]. In this model, peripheral injection of CRH receptor antagonists was able to significantly reduce intestinal secretion and inflammation. In accordance with this, genetically CRH-deficient mice showed diminished intestinal responses to *C. difficile* toxin A[10], and RNA interference to silence

CRH expression in the ileum ablated the toxin A-induced inflammatory responses, such as epithelial damage, mucosal edema, and neutrophil infiltration[11]. Collectively, these results indicate that peripheral CRH acts as a pro-inflammatory mediator in intestinal inflammation.

Considering the involvement of peripheral CRH in intestinal inflammation, together with the putative role of intestinal inflammation in the development of visceral hypersensitivity, we hypothesized that increased peripheral CRH might be involved in post-inflammatory visceral hypersensitivity. We tested this hypothesis using a rat model that shows elevated nociceptive responses to luminal distension 7 d after acetic acid-induced colitis without histological and biochemical signs of overt gut inflammation[12]. In this rat model, we found that high levels of peripheral CRH were related to post-inflammatory visceral hypersensitivity.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 270-310 g, were housed in a colony room maintained under a 12-h light/dark cycle with a room temperature of 22 ± 1℃ and humidity of 65%-70%. Water and food were available *ad libitum*. Before the intracolonic instillation of chemicals, rats were fasted for 18 h. Colitis was induced by instillation of 1 mL 4% acetic acid into the colon. After 30 s, saline (1 mL) was instilled to flush the lumen. Control animals received saline only (1 + 1 mL). All experimental protocols in this study were reviewed and approved by the Animal Care and Use Committee of Seoul National University.

Myeloperoxidase (MPO) activity

A segment of distal colon (for MPO activity assay) and trunk blood (for plasma CRH level assay) were collected from rats sacrificed by cervical dislocation, following anesthesia with tiletamine/zolazepam (50 mg/kg) between 2:00 and 4:00 pm. The animals had not undergone any treatment (e.g. colorectal distension) other than intracolonic saline or acetic acid instillation. The mucosal layer of the distal colon was stripped off from the segment and minced in 1 mL 50 mmol/L potassium phosphate buffer (pH 6.0) that contained 14 mmol/L hexadecyltrim ethylammonium bromide (Sigma-Aldrich, St. Louis, MO, USA). After homogenization and sonication, the lysates were frozen and thawed three times, then centrifuged for 2 min under 4℃ at 15000 × *g*. Aliquots of the supernatants were mixed with o-dianisidine-HCl (Sigma-Aldrich) and 0.0005% H_2O_2. MPO activity was calculated from the rate of absorbance change, during 1 min, at 460 nm using the extinction coefficient for oxidized o-dianisidine (1.13×10^4/mol/L/cm) and expressed as units/mg of tissue protein quantified by the Bradford method. The enzyme unit was defined as the conversion of 1 μmol of H_2O_2 per min at 25℃.

Plasma CRH level

Blood samples collected in ice-cold EDTA-coated tubes were centrifuged at 1600 × *g* for 15 min at 4℃. Plasma obtained after centrifugation was transferred into sterile microcentrifuge tubes and stored at -70℃ until analyzed. As it is known that the CRH-binding protein interferes with plasma CRH level estimation, we extracted CRH from plasma using ice-cold methanol[13]. Specifically, plasma samples were mixed and incubated with methanol for 15 min at 4℃, and then centrifuged at 2000 × *g* for 20 min at 4℃. The supernatants were transferred to another set of tubes. The pellets were washed with 0.2 mL ice-cold methanol and centrifuged at 2000 × *g* for 15 min at 4℃. The supernatants were pooled and dried by blowing heated air. The extracts were then reconstituted with assay buffer (one-third of original plasma volume) included in the CRH enzyme immunoassay kit (Phoenix Pharmaceuticals, Belmont, CA, USA). Each sample was processed in duplicate and the final data were expressed as pg CRH/mL plasma.

Visceral nociceptive behavior to colorectal distension

Eighteen hour-fasted rats were lightly anesthetized with ether, and a disposable silicon balloon urethral catheter was carefully inserted intra-anally until the tip of the balloon was 4 cm proximal to the anus. While animals were fully awake, ascending-limits phasic distensions (0.2, 0.4, 0.6, 0.8 and 1 mL) were applied for 30 s every 4 min, and the distension-induced abdominal withdrawal reflex (AWR) was scored by an experimenter who was blind to the treatments (0, no behavioral response to distension; 1, brief head movements followed by immobility; 2, contraction of abdominal muscle without lifting of abdomen; 3, lifting of abdomen; and 4, body arching and lifting of pelvic structure)[14]. The overall difference in the visceral nociceptive responses between groups was determined by taking the area under the curve (AUC) calculated as the sum of the responses plotted against the distension volume using the trapezoidal rule.

Chemicals

Astressin (Bachem, Budendorf, Switzerland) and rat/human CRH (American Peptide, Sunnyvale, CA, USA) were dissolved in sterilized saline. Stock aliquots of these peptides were kept at -70℃, and diluted to a final concentration before use.

Statistical analysis

Data are presented as mean ± SE, with n the number of animals. The difference between two groups was statistically analyzed using the Mann-Whitney *U* test (MWU test) at the $P < 0.05$ significance level. Comparisons between three or more groups were performed with Kruskal-Wallis test (KW test), followed by Dunnett's procedure.

RESULTS

To quantify the extent of inflammation, we measured the MPO activity from colonic mucosa. As shown in Figure 1A, MPO activity was dramatically increased in rats at 2 d after induction of colitis (21.0 ± 3.4 *vs* 146.0 ± 45.5 U/mg, $n = 10$ in each group, $P < 0.01$). After 7 d, MPO activity returned to normal (22.0 ± 1.2 U/mg, $n = 10$), indicating a substantial resolution of inflammation. Plasma CRH level was also elevated about twofold in rats at 2 d (333.5 ± 19.7

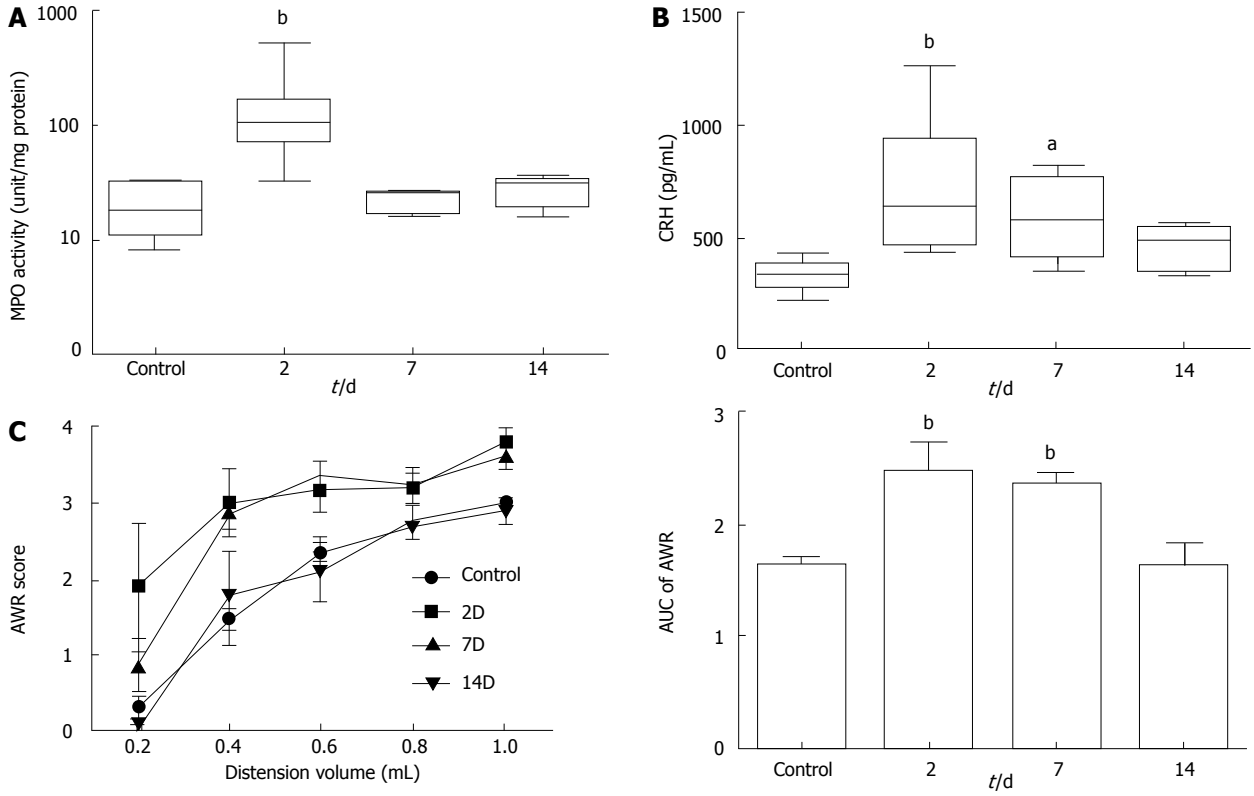


Figure 1 Colitis-induced changes in colonic mucosal MPO activity (**A**), plasma level of CRH (**B**), and visceral nociceptive AWR (**C**). In panel **A** and **B**, the upper and lower whiskers indicate the maximum and minimum values, respectively. Box lines represent the 25th (bottom), 50th (middle) and 75th (top) percentile value; (**C**) The area under the curve (AUC) calculated from the stimulus-response plot is illustrated as a bar graph on the right side. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control by MWU test after KW test ($P < 0.01$).

vs 740.1 ± 88.8 pg/mL, $n = 10$ in each group, $P < 0.01$). Unlike MPO activity, however, the increase in plasma CRH level was maintained in rats at 7 d (587.6 ± 61.1 pg/mL, $n = 8$, $P < 0.05$ from control). It was decreased down to the control level 14 d after induction of colitis (Figure 1B).

Figure 1C demonstrates the response pattern of each experimental group to colorectal distension. We could not detect any difference between the day-matched control rats, thus we pooled the data obtained from control animals. At 2 d after induction of colitis, the animals showed enhanced AWR response, which resulted in a significant increase in the AUC of the stimulus-response plots (1.6 ± 0.1, $n = 15$ *vs* 2.5 ± 0.3, $n = 5$, $P < 0.01$). Rats at 7 d also clearly showed visceral hypersensitivity (2.4 ± 0.1, $n = 8$, $P < 0.01$ from control). Fourteen days after colitis induction, the animals were found to have recovered from the post-inflammatory visceral hypersensitivity.

A similar time course for increased plasma CRH level and enhanced visceral nociceptive behavior, i.e., AWR to colorectal distension, raised the possibility that the sustained increase in peripheral CRH might account for the post-inflammatory visceral hypersensitivity. We further tested this possibility by intraperitoneal administration of CRH receptor antagonist astressin (30 μg/kg), 30 min before colorectal distension. The dose of astressin was chosen based on its ability to prevent defecation and c-Fos expression, which was induced by peripherally administered CRH in other studies[15,16]. Astressin did not change the AWR behavior in control rats (1.7 ± 0.1 *vs* 1.7 ± 0.1, $n = 7$ in each group). However, it significantly decreased the AUC of AWR score in rats at 7 d (2.3 ± 0.2, $n = 7$ *vs* 1.8 ± 0.1, $n = 6$, $P < 0.05$), which indicated that endogenous peripheral CRH mediated the post-inflammatory visceral hypersensitivity (Figure 2A).

We further tested this idea by peripherally injecting exogenous CRH to naïve rats (1, 3 and 10 μg/kg, i.p., $n = 5$ in each group) 30 min before colorectal distension. As shown in Figure 2B, rats that received 3 or 10 μg/kg CRH developed visceral hypersensitivity to colorectal distension. Astressin, administered 15 min before the injection of CRH, showed a tendency to inhibit CRH-induced visceral hypersensitivity in naïve rats (2.3 ± 0.2 *vs* 1.8 ± 0.1, $n = 5$ in each group, $P = 0.07$).

DISCUSSION

The present study provides evidence that suggests increased peripheral CRH underlies the post-inflammatory visceral hypersensitivity. First, visceral hypersensitivity could be observed in rats only at the experimental time points when plasma CRH level was higher than the control level. We found a significant increase in plasma CRH in rats at 2 and 7 d after colitis induction. The level of plasma CRH was back to normal after 14 d. Interestingly, rats at 2 d and 7 d, but not 14 d, showed enhanced AWR behavior in response to colorectal distension. This correlation between sustained CRH increase and visceral hypersensitivity has also been implicated in another rat colitis model. Two different studies have revealed that rats that have recovered from trinitrobenzene sulphonic acid-induced colitis show visceral hypersensitivity to colorectal distension at 28 d after colitis induction[17], and a sustained increase in CRH

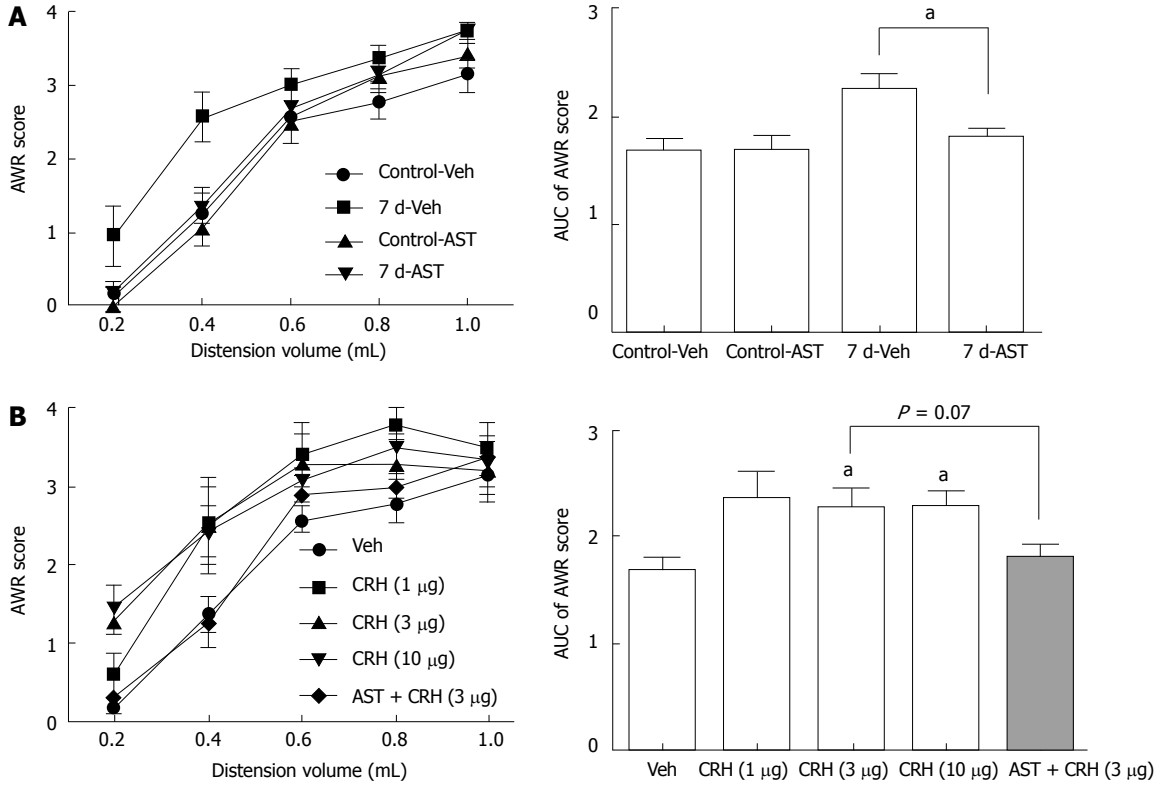


Figure 2 Effect of CRH receptor antagonist astressin (**A**) and CRH (**B**) on the colorectal distension-induced AWR behavior. In each panel, the bar graphs on the right side represent the AUC of the stimulus-response plot. Astressin (AST, 30 μg/kg) and CRH (1, 3 and 10 μg/kg) were administered intraperitoneally. The same amount of saline was given as a vehicle (Veh). $^{a}P < 0.05$ *vs* the paired group in (**A**) or the Veh group in (**B**) by MWU test after KW test ($P < 0.05$).

mRNA expression in hypothalamic paraventricular nucleus at d 30[18]. These two independent reports collectively suggest a role of central CRH in the post-inflammatory visceral hypersensitivity. Considering that the continuous CRH increase in plasma during sustained stress is derived mainly from the hypothalamus[19], it is also likely that the sustained increase in hypothalamic CRH heightens the level of plasma CRH acting peripherally after the resolution of colitis. It will be necessary in future studies to examine the source of increased plasma CRH in our model of post-inflammatory visceral hypersensitivity. Along with the paraventricular nucleus neurons in the hypothalamus, local immune cells resident in the gut may be the sources of the increased CRH in the post-colitis condition.

The second piece of evidence is that astressin, a CRH receptor antagonist that cannot cross the blood-brain barrier, alleviated the visceral hypersensitivity in rats at 7 d when administered intraperitoneally, whereas it had no effect on the AWR behavior in control rats. These results show that endogenous peripheral CRH plays a crucial role only in the hypersensitive visceral nociception in post-colitis condition, but not in normal visceral nociception upon colorectal distension. It is interesting that transgenic mice deficient in CRH1 receptor are less sensitive to colorectal distension than wild-type mice, which indicates that activation of CRH1 receptor is crucial, even in normal visceral nociception in mice[20]. The discrepancy between the results of this study and our current results might be explained by the species difference (mice *vs* rats) and/or the difference in the level of CRH receptor blockade, because knocking out the CRH1 receptor gene eliminates both central and peripheral targets of CRH.

Our third piece of evidence is that peripheral administration of CRH mimicked the post-inflammatory visceral hypersensitivity in naïve rats. How can peripheral CRH render animals more sensitive to colorectal distension? Inflammatory substances are known to sensitize visceral afferents, which results in a decrease in the response threshold, as well as an increase in response magnitude[21]. The sensitization of visceral afferents is regarded as a fundamental mechanism of visceral hypersensitivity[22]. Peripheral CRH has been shown to stimulate mast cells that secrete numerous inflammatory substances including histamine, cytokines, proteases and eicosanoids[23,24]. Interestingly, mast cells have long been implicated to be involved in IBS and visceral hypersensitivity. An increased number of mast cells have been noted in the mucosa of IBS patients[25-28], and the correlation between mast cells and visceral hypersensitivity has been shown in experimental animals. Gue *et al*[29] have reported that the mast-cell stabilizer doxantrazole effectively reduces restraint stress-induced visceral hypersensitivity in rats. McLean *et al*[30] have also reported that, in *Nippostrongylus brasiliensis*-infected rats, an increase in mucosal mast cells was observed at 30 d but not at 90 d post-infection, and that the hypersensitive depressor response to jejunal distension was observed only at 30 d post-infection. In our previous study using the same animal model used in the present study, we found that the number of degranulated mucosal mast cells was higher in rats at 7 d after colitis induction, and that doxantrazole inhibited the post-inflammatory visceral hypersensitivity

in these rats[31]. Therefore, it can be speculated that mast cell activation by peripheral CRH accounts for its ability to induce visceral hypersensitivity. It would be of interest to examine if doxantrazole can prevent the development of visceral hypersensitivity caused by exogenously administered CRH.

Our present findings support the idea that peripheral CRH receptor can be a target for treating IBS symptoms. Recent studies have demonstrated that administration of alpha-helical CRH (9-41), a CRH receptor antagonist, significantly improves gastrointestinal motility, and reduces abdominal pain and anxiety evoked by rectal electrical stimulation in IBS patients[32]. The same antagonist has also been reported to blunt changes in the electroencephalogram (EEG) power spectra upon colonic distention, and almost normalize EEG activity in IBS patients[33]. The effectiveness of CRH receptor antagonist on the IBS-like symptoms, especially visceral hypersensitivity, has also been reported in animal studies. Antalarmin, a CRH1 receptor antagonist, prevents visceral hypersensitivity induced by repeated colorectal distension in female rats[34]. Peripherally administered CRH1 receptor antagonist CP-154 526, which can act centrally by freely crossing the blood-brain barrier, inhibits acute stress-induced visceral hypersensitivity in maternally separated rats[35].

In summary, we found that rats with visceral hypersensitivity after resolution of colitis showed sustained increase in plasma CRH level and that a CRH receptor antagonist inhibited the symptom, which suggests that the increased peripheral CRH mediates the post-inflammatory visceral hypersensitivity, and that CRH receptor can be a target for alleviating this symptom.

COMMENTS

Background

Intestinal inflammation is thought to induce persistent changes in gut function, and indeed, visceral hypersensitivity often develops after intestinal inflammation. However, it is still not well understood what mediates the heightened sensitivity of the gut after resolution of the inflammation.

Research frontiers

IBS is a disorder whose hallmark symptom is visceral hypersensitivity, which is believed to be caused or exacerbated by stress, and corticotropin releasing hormone (CRH) is a pivotal central mediator of the stress response. Therefore, the role of CRH in this bowel disorder is gaining interest. It is also becoming clear that peripheral CRH expression is increased in intestinal inflammation, which raises the possibility that peripheral CRH might be involved in visceral hypersensitivity developed after intestinal inflammation.

Innovations and breakthroughs

Using a rat model of acute colitis as a model of post-inflammatory visceral hypersensitivity, the present study showed the correlation between the level of peripheral CRH and the development of visceral hypersensitivity after experimental colitis. Here, it was also shown that peripheral administration of CRH receptor antagonist and CRH itself could inhibit and mimic the post-inflammatory visceral hypersensitivity, respectively.

Applications

The findings from this study support the idea that peripheral CRH receptor can be a therapeutic target for treating visceral hypersensitivity in IBS.

Terminology

Visceral hypersensitivity refers to increased sensation of stimuli in the gut, which is defined by lowered threshold for visceral pain/discomfort elicited by intraluminal mechanical/chemical stimuli.

Peer review

The design of the controls was rational and reliable. The statistical methods used were appropriate. The results provided sufficient evidence to arrive at the conclusions. The discussion was well organized, and valuable conclusions are provided.

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RAPID COMMUNICATION

Treatment of rectal prolapse in children with cow milk injection sclerotherapy: 30-year experience

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Abstract

AIM: To evaluate the role and our experience of injection sclerotherapy with cow milk in the treatment of rectal prolapse in children.

METHODS: In the last 30 years (1976-2006) we made 100 injections of sclerotherapy with cow milk in 86 children. In this study we included children who failed to respond to conservative treatment and we perform operative treatment.

RESULTS: In our study we included 86 children and in all of the patients we perform cow milk injection sclerotherapy. In 95.3% (82 children) of patients sclerotherapy was successful. In 4 (4.7%) patients we had recurrent rectal prolapse where we performed operative treatment. Below 4 years we had 62 children (72%) and 24 older children (28%). In children who needed operative treatment we performed Thiersch operation and without any complications.

CONCLUSION: Injection sclerotherapy with cow milk for treatment rectal prolapse in children is a simple and effective treatment for rectal prolapse with minimal complications.

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Key words: Rectal prolapse; Sclerotherapy with cow milk; Children

Peer reviewer: Peter Laszlo Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

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INTRODUCTION

Prolapse of the rectum is a herniation of the rectum through the anus[1]. The herniation may be mucosal and may involve only a small ring of mucosa or involve all layers of the rectum (procidentia). These conditions are most common from 3-5 years of age and usually first detected by the childs' parents and usually spontaneously reduce. Parents often provide history of a dark red mass protruding from the childs' anus and the child appears to be pain free[2]. Prolapse of the rectum, which involve only the mucosa, is the least serious form and is most common in the pediatric population[3].

The most common form of rectal prolapse is idiopathic. At this age a child's muscle mass is not well developed and the rectum is loosely adherent to the underlying muscles. These children also spend long periods straining to evacuate their rectum with hips and knees flexed while sitting on a children's "potty"[4]. Children with conditions such as rectal polyps, diarrhea, malnutrition, worms, proctitis, ulcerative colitis congenital megacolon, Ehlers-Danlos syndrome and cystic fibrosis also may have rectal prolapse. Rectal prolapse is common in children with exstrophy of bladder and myelomeningocoele or in similar conditions associated with weakness pelvic musculature or its innervation[5].

Rectal prolapse often occurs with defecation or crying and reduces spontaneously. Failure to reduce may lead to venous stasis, edema and possibly ulcerations. When prolapse is present it is not possible to insert fingers in the space between the prolapsed bowel and anus. The prolapsed bowel may be grasped with lubricated fingers and pushed back in by the parents. If rectal prolapse persists for a long time the bowel becomes edematous and firm steady pressure for several minutes may be necessary to reduce the swelling and allow for reduction[6]. After reduction digital rectal examination is necessary to verify complete reduction. If prolapse immediately recurs, reduction is again necessary and the buttocks strapped together with a single band of adhesive tape for several hours[7].

Most patients with children's rectal prolapse do not require any specific form of treatment; however, it is necessary to prevent excessive straining. Treatment should be directed at dietary correction of constipation, proper toilet training, and at elimination of any underlying disturbance such as parasitic infection, polyps or diarrhea. Oral administration of stool softeners and having the child defecate with feet off the floor may be helpful[8]. Prolonged sessions on the toilet should be discouraged[9]. When parents observe rectal prolapse, reduction of protrusion is aided by pressure with warm compresses and gently push it into the rectum. An immediate replacement of rectal prolapse is necessary to avoid swelling and possible later difficulty in reduction[10]. If edema of the rectum persists, firm steady pressure of the fingertips for several minutes may be necessary to reduce swelling and permit reduction of the prolapse[11]. Surgical procedures may be required for recurrent rectal prolapse refractory to conservative measures[12]. If repetitive attempts at medical therapy have failed and conservative treatments also failed, and the prolapse persists, submucosal injection of sclerosans into the rectal ampula should be done. Children with recurrence after injection of sclerosans require surgical treatment[13].

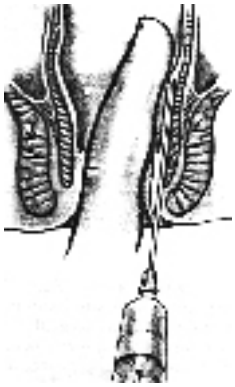


Figure 1 Cow milk is injected along the rectal submucosa.

Table 1 Rectal prolapse in the last 30 years (n = 483 children)

Treatment	n
Conservative	397
Sclerotherapy	86
One injection	76
Two injections	6
Three injections	4
Operative	4

MATERIALS AND METHODS

Clinical features

In children with rectal prolapse, before any treatment, we performed sweat chloride test to rule out cystic fibrosis since the incidence of rectal prolapse in patients with cystic fibrosis ranges from 11%-23%[14]. We also obtained patients' stool for ova and parasites. Rectal prolapse has been associated with *Escherichia coli*, antibiotic-associated colitis, *Entamoeba histolytica, Giardia, Salmonella, Shigella,* and *Trichuris species*. Children with recurrent rectal prolapse, with no found underlying causes, should undergo proctosigmoidoscopy to rule out rectal polyps or other rectal lesions[14]. If found, the underlying cause of the prolapse should be treated. Most patients with children's rectal prolapse do not require any specific form of treatment, however, it is necessary to prevent excessive straining. Prolonged sessions on the toilet should be discouraged[15].

In the last 30 years (1976-2006) we had 483 patients with rectal prolapse. Three hundred and ninety seven patients with children's rectal prolapse did not require any specific form of treatment, it is necessary to prevent excessive straining. In this study we included children who failed to respond to conservative treatment and we performed sclerotherapy. In 86 patients repetative attemps at medical therapy failed and conservative treatment also failed and the prolapse persisted; here submucosal injection of sclerosans into the rectal ampula should be done.

Methods

We made 100 injections of sclerotherapy with cow milk in 86 patients. In this study we used conservative treatments and also performed operative treatments. We included 86 children and in all of patients we performed operative treatment. In all children we excluded allergic reactions to cow milk. In 95.3% (82 children) of patients the sclerotherapy was successful and in 4 (4.7%) children we performed operative treatment. Below 4 years we had 62 children (72%) and 24 older children (28%). The youngest boy was 8 mo. old and oldest was 8 years old. The mean age of the patients treated with sclerotherapy was 3.6 years. In 4 children, who needed operative treatment for recurrent rectal prolapse after three cow milk injections of sclerotherapy, we performed the Thiersch operation without complications. The youngest patient with Thiersch operation was 4 years old and oldest girl was 8 years old, the mean age of the patients was 6.2 years.

The technique of sclerotherapy is not difficult, but the surgeon should have experience with this method. The rectum is emptied with suppository and not with an enema. The patient is in the lithotomy position. A long needle is attached to an injection syringe inserted through the skin just outside the mucocutaneos junction and guided to the proper position using a finger in the rectum[16]. Two or three milliliters of cow milk are injected along the rectal submucosa at the depth of 4-5 cm at four different quadrants (Figure 1). In 76 patients, we perform one cow milk injection, in 6 patients two cow milk injections, and in 4 patients three cow milk injections. In these 4 patients with three cow milk injections we performed operative surgical treatment (Table 1). This technique, submucosal injection of sclerosans into the rectal ampula, is designed to produce a perirectal inflammatory reaction to induce scarring and prevent prolapse[17]. Before application of cow milk, the milk was boiled for 10 min. Afterwards, the milk was cooled and application was possible.

In children with recurrent rectal prolapse, after three cow milk injection sclerotherapies, we performed the Thiersch procedure for rectal prolapse. The Thiersch

technique consists of a small incision in the perianal skin at 6 and 12 o'clock[18]. We performed the Thiersch operation with wire in two patients and two patients with a nonabsorbable suture. Through anterior and posterior skin incisions a wire is passed around the anal canal just proximal to the external anal sphincter and tied at an appropriate tension (tie wire/nonabsorbable suture just tight enough to prevent rectum prolapsing and just loose enough to let pass stool)[19]. This wire is tied over a 12 (infant) to 20 (adolescent) Hegar dilator or surgeon's finger placed into the rectum. The therapy is effective to mechanically prevent rectum prolapsing, but it does not treat the underlying disorder. The purpose of this procedure is to keep the rectum from prolapsing by restricting the size of the anal lumen[20].

RESULTS

In the last 30 years (1976-2006) we made 100 injection sclerotherapies with cow milk in 86 children. In this study, we included children who failed to respond to conservative treatment and then we performed operative treatment.

In our study we included 86 children and in all patients we performed cow milk injection sclerotherapy. In 95.3% (82 children) of patients the sclerotherapy was successful and in 4 children with recurrent rectal prolapse we performed operative treatment. We had 62 children (72%) younger than 4 years and 24 older children (28%). The mean age for children with sclerotherapy was 3.6 years. In children who needed operative treatment we performed the Thiersch operation and no children had complications. The mean age of the children with the Thiersch operation was 6.2 years.

When we performed cow milk sclerotherapy every patient had a fever for 2 d and no antibiotics were administrated. We had only one patient who had inflammation in the place where milk was performed and incision was performed. After the Thiersch operation, fortunately, the wire was not rejected by the body and patients did not have enough scar tissue formed to improve control of the anus and to delay the return to the prolapse for months or years after removal of the wire or nonabsorbable suture. The wire was removed 6 mo after the Thiersch operation.

DISCUSSION

There is no optimal or standard procedure for treatment of rectal prolapse[21]. Although in excess of many different operations which have been described so far, some more popular than the others, we performed two procedures in our children's hospital[22].

Most patients with children's rectal prolapse do not require any specific form of treatment; however, it is necessary to prevent excessive straining. Treatment should be directed to dietary correction of constipation, to proper toilet training, and to the elimination of any underlying disturbance such as parasitic infection, polyps or diarrhea. Oral administration of stool softeners and having the child defecate with feet off the floor may be helpful. Prolonged sessions on the toilet should be discouraged.

If conservative treatment was not effective, cow milk injection sclerotherapy is simple and should be considered as the first line treatment of recurrent prolapse. We did not use a sclerosant agent such as 30% saline or phenol in almond oil because cow milk as sclerosing agent is inexpensive, easy for application, and the procedure is safe with no side effects[23]. We had only one complication involving inflammation of the perianal tissue. This procedure involves a 2 d stay in hospital after sclerotherapy.

In children with recurrent rectal prolapse following three cow milk injection sclerotherapies, we performed the Thiersch procedure for rectal prolapse[24]. In 4 children we performed operative treatment, the Thiersch operation. This procedure involves a 2 d stay in hospital after the operation. We did not have any complications after the procedure and the wire was removed 6 mo after the operation[25]. All patients, after the operation, did not have recurrent rectal prolapse.

Rectal prolapse is an intussusception of the rectum, which may be categorized as mucosal or complete. Mucosal prolapse involves protrusion of the mucosa only with the muscular layers of the rectum remaining in place[26]. Complete rectal prolapse or rectal procidentia involves full-thickness protrusion of the rectum through the anus.

Most patients with children's rectal prolapse do not require any specific form of treatment, but it is necessary to prevent excessive straining. Treatment should be directed at dietary correction of constipation, to proper toilet training, and to the elimination of any underlying disturbance such as parasitic infection, polyps or diarrhea. Oral administration of stool softeners and having the child defecate with feet off the floor may be helpful. Prolonged sessions on the toilet should be discouraged.

Surgical procedure may be required for recurrent rectal prolapse refractory to conservative measures. If repetitive attempts at medical therapy have failed and conservative treatment has also failed, and the prolapse persists, submucosal injection of sclerosans into the rectal ampula should be done[27]. Cow milk as a sclerosing agent did not cause any complications. Injection sclerotherapy is simple and effective way to treatment for rectal prolapse in children[28]. Children with recurrence after three injections of sclerosing agent require operative surgical treatment[29].

In the last 30 years (1976-2006) we have performed 100 injection sclerotherapies with cow milk in 86 children. In 95.3% (82 children) of patients the sclerotherapy was successful and in 4 children we performed operative treatment. In children who needed operative treatment we performed the Thiersch operation and no children developed complications[30].

Both surgical treatments (cow milk sclerotherapy and the Thiersch operation) which we performed in our hospital in the last 30 years were very effective, inexpensive, easily available with only 2 d stay in hospital. The results were very good after cow milk sclerotherapy (95.3% patients without recurrence), and 4 patients after the Thiersch procedure were without recurrent rectal prolapse.

COMMENTS

Background

Prolapse of the rectum is a herniation of the rectum through the anus. The herniation may be mucosal and may involve only a small ring of mucosa or involve all layers of the rectum (procidentia).

Research frontiers

Most patients with children's rectal prolapse do not require any specific form of treatment; however, it is necessary to prevent excessive straining. Treatment should be directed at dietary correction of constipation, to proper toilet training, and to the elimination of any underlying disturbance such as parasitic infection, polyps or diarrhea.

Innovations and breakthroughs

Cow milk as a sclerosing agent is inexpensive, easy for application, the procedure is safe with no side effects. Injection sclerotherapy is a simple and effective way to treat rectal prolapse in children. Submucosal injection of sclerosans into the rectal ampula is designed to produce perirectal inflammatory reaction to induce scarring and prevent prolapse. Before cow milk application, the milk was boiled for 10 minutes. After that, the milk was cooled and application was possible.

Applications

In 76 patients (88.3%) we perform one cow milk injection and 95.3% patients were without recurrence. Cow milk is inexpensive, easy for application and without contraindications, except cow milk allergy.

Terminology

Rectal prolapse: Herniation of the rectum through the anus. Sclerotherapy: Submucosal injection of sclerosans into the rectal ampula, is designed to produce perirectal inflammatory reaction to induce scarring and prevent prolapse.

Peer review

The authors reported their long term experience with the treatment of rectal prolapse in children. In relapsing cases they reported a high success rate with cow milk sclerotherapy. This is an interesting manuscript.

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RAPID COMMUNICATION

Diagnosis of Crohn's disease in India where tuberculosis is widely prevalent

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Abstract

AIM: To define the parameters that positively predict diagnosis of Crohn's disease (CD) and differentiate it from gastrointestinal tuberculosis (GITB).

METHODS: This prospective study over 3 years was carried out in the consecutive Indian patients with definite diagnosis of CD and equal numbers of patients with definite diagnosis of GITB. Demographic, clinical, laboratory, morphological and histological features were noted in all the patients. Serological tests such as p-ANCA, c-ANCA, IgA ASCA and IgG ASCA, were performed. Endoscopic biopsy and/or surgical tissue specimens were subjected to smear and culture for acid-fast bacilli (AFB) and tissue polymerase chain reaction for tuberculosis (TB PCR). Diagnosis of CD and GITB was based on the standard criteria. Data were analyzed using univariate Chi-square test and multiple logistic regression (MLR).

RESULTS: The study is comprised of 26 patients with CD (age 36.6 ± 8.6 year, male:female, 16:10) and 26 patients with GITB (age 37.2 ± 9.6 year, male:female, 15:11). The following clinical variables between the two groups (CD *vs* TB) were significant in univariate analysis: duration of symptoms (58.1 ± 9.8 *vs* 7.2 ± 3.4 mo), diarrhoea (69.2% *vs* 34.6%), bleeding per rectum (30.7% *vs* 3.8%), fever (23.1% *vs* 69.2%), ascites (7.7% *vs* 34.6%) and extra-intestinal manifestations of inflammatory bowel disease (61.5% *vs* 23.1%). Of these, all except ascites and extra-colonic manifestations were found statistically significant by MLR. Accuracy of predicting CD was 84.62% based on the fever, bleeding P/R, diarrhoea and duration of symptoms while it was 63.4% when histology was reported as inflammatory bowel disease and 42.3% when there was recurrence of disease after surgery. Accuracy of predicting GITB was 73.1% when there was co-existing pulmonary lesions and/or abdominal lymphadenopathy; 75% when tuberculosis was reported in histology; 63.4% when granuloma was found in histology; 82.6% when TB PCR was positive; and 61.5% when smear and/ or culture was positive for AFB. Serological test was not useful in differentiation of CD from GITB. Positivity rates for CD and GITB were: p-ANCA- 3.8% and 3.8%, c-ANCA- 3.8% and 0%, IgA ASCA- 38.4% and 23.1%, and IgG ASCA- 38.4% and 42.3%, respectively.

CONCLUSION: Simple clinical parameters like fever, bleeding P/R, diarrhoea and duration of symptoms have the highest accuracy in differentiating CD from GITB.

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Key words: Crohn's disease; Gastrointestinal tuberculosis; Differential diagnosis; Inflammatory bowel disease; Anti-neutrophil cytoplasmic antibody; Anti-*Saccharomyces Cerevisiae* antibody; Tissue polymerase chain reaction; *Mycobacterium Tuberculosis*; Acid-fact bacilli

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INTRODUCTION

In Western countries, the incidence of inflammatory bowel disease (IBD) has progressively increased since 1935[1]. Ulcerative colitis (UC) has peaked earlier than Crohn's disease (CD)[1,2]. Although CD has been increasing worldwide[3], it is generally believed to be rare in developing countries like India[4]. Indian migrants in Western countries have shown increased incidence of IBD, more so in the second generation of immigrants[1,5]. In India, the incidence of UC has significantly increased over the last three decades, whereas the incidence of CD increased progressively in the last decade[4,6-15]. Tuberculosis (TB) remains widely prevalent in developing countries such as India. Also, it is being increasingly encountered in industrialized nations due to

pandemic of human immunodeficiency virus infection and trans-global migration[16-19].

There is a close resemblance in clinical, radiological, endoscopic, surgical and histological features of CD and gastrointestinal tuberculosis (GITB), thus differential diagnosis of these two conditions remains a major challenge to clinicians[7-9,12,19-23]. In India, as more and more cases of CD are being recognized, the definite diagnosis of CD or GITB becomes increasingly important[8,10]. In case of misdiagnosis of GITB, unnecessary anti-tuberculosis therapy (ATT) poses a risk of toxicity and treatment of the primary disease, i.e. CD is delayed. In contrast, treatment with steroids alone (for CD) can be disastrous, if diagnosis of GITB is missed. As response to empirical ATT is considered to be a favouring point for final diagnosis of TB, emergence of multi-drug resistant TB can further complicate the issue by decreasing the therapeutic response even in presence of TB. All these situations highlight the need to establish the diagnosis of either CD or GITB, before starting any form of empirical treatment.

In a recently published study with an exhaustive search for histological and microbiological proof for GITB, definite diagnosis could be established only in 66.5% of cases[10]. In other previous series, attempts are made to differentiate CD and GITB on histopathology of endoscopic or surgical specimens[7,12,22-24]; but no single feature can definitely exclude TB. Recent advances in armamentarium of diagnosis of CD and TB are serology tests, such as anti-neutrophil cytoplasmic antibody (ANCA) and anti-*Saccharomyces Cerevisiae* antibody (ASCA), and tissue polymerase chain reaction (PCR) for *Mycobacterium Tuberculosis* (TB PCR)[25-27]. In one study by our centre, TB PCR was found highly specific for diagnosis of GITB, but lacked sensitivity[11]. In another study at our centre, ASCA was not found useful in differentiating CD from GITB[28].

Thus, it is very difficult to differentiate CD from GITB based on index evaluation of a patient. No single diagnostic criterion can define CD. Follow-up evaluations and treatment response are also important to reach the diagnosis. We have designed a study to define the accuracy of various parameters which can predict diagnosis of either CD or GITB at index evaluation.

MATERIALS AND METHODS

Study design

A prospective data analysis was performed in patients with definite diagnosis of CD and GITB. These data were collected prospectively during the study period.

Patient evaluation

During the study period from January 2002 to December 2004, all the patients with suspected diagnosis of CD and GITB were prospectively evaluated for the following parameters (details wide infra): demographic, clinical, laboratory, morphology, histology, microbiology, serology and treatment response at follow-up evaluation for next one year.

Demographic (age, sex and ethnicity), clinical (chronic diarrhoea, hematochezia, features of intestinal obstruction, fever, perianal involvement, extra-intestinal manifestations, abdominal pain, ascites, weight loss, growth retardation, abdominal lump and duration of symptoms, long duration: > 1 year) and laboratory features (anaemia, hypoproteinemia and erythrocyte sedimentation rate) were noted in all the patients.

Morphological findings of intestine during endoscopy (endoscopic evaluation was done in all by the first author): skip lesions, deep ulcers, aphthous ulcers, fissures, fistula, cobble-stone appearance, stricture and site of involvement.

Histological findings were evaluated using paraffin-embedded tissue specimens after staining with hematoxylin and eosin. Histology of all the specimen was reported by an experienced gastrointestinal pathologist, who was completely unaware of clinical, morphological, microbiological and serological findings. The histological features were as follows: presence of granuloma, characteristics of granuloma (caseation or confluence), ulceration of surface epithelium, appearance of crypts, presence of lymphoid aggregates and site and type of inflammatory infiltrate. Presence of large, dense and confluent mucosal or submucosal caseating granuloma, > four sites of granulomatous inflammation, caseation, band of epitheloid histiocytes in ulcer base and granulomatous inflammation in cecum favoured histopathological diagnosis of TB; whereas presence of small, discrete and loose mucosal non-caseating granuloma, mucosal changes distant to sites with granuloma, focal crypt-related inflammation and granuloma in sigmoid or rectum favoured histopathological diagnosis of CD.

Biopsy specimens (endoscopic, at least 6 biopsy samples, or surgical) were subjected to Ziehl-Neelsen and fluorescence smear for AFB, Bactec culture for AFB and TB PCR. Paraffin-embedded biopsy specimens were tested for detection of *Mycobacterium Tuberculosis* complex by the Gen-Probe (USA) amplified *Mycobacterium Tuberculosis* direct test by PCR assay[11]. The following serological tests were done in all the patients: IgG perinuclear-ANCA (p-ANCA), *in vitro* detection of IgG class of antibodies to myeloperoxidase using quantitative ELISA (MPO antibodies ELISA kit, Genesis diagnostics, UK); IgG cytoplasmic-ANCA (c-ANCA), *in vitro* detection of IgG class antibodies to serine protease-3 (PR3 antibodies ELISA kit, Genesis diagnostics, UK); IgA ASCA, detection of IgA anti-Saccharomyces Cerevisiae antibodies using quantitative ELISA (IgA ASCA ELISA kits, Genesis diagnostics, UK); IgG ASCA, detection of IgG anti-Saccharomyces Cerevisiae antibodies using quantitative ELISA (IgG ASCA ELISA kits, Genesis diagnostics, UK). Titres were considered positive if they were above the upper limit of normal range, i.e. for p-ANCA 0-4 μ/mL, for c-ANCA 0-4 μ/mL, IgA ASCA 0-10 μ/mL and for IgG ASCA 0-10 μ/mL.

All the surgical histological specimens of the patients who underwent intestinal surgeries in the past were re-examined using the above mentioned methods.

During the 1-year follow-up, all the patients were evaluated at regular interval with clinical, laboratory and imaging parameters. Endoscopic evaluations with histological and microbiological studies were performed at least twice (at 6 mo and at 1 year) during the follow-up.

Diagnostic criteria

In presence of demonstrable lesion in gastrointestinal tract,

diagnosis of GITB was established based on the following criteria: (1) presence of caseating granuloma on histology of diseased tissue (intestine, peritoneum or lymph nodes); (2) demonstration of acid-fast bacilli (AFB) on smear or on histological section; (3) positive culture for AFB; (4) histological or microbiological confirmed TB at extra-intestinal site; and (5) positive TB PCR. For the definite diagnosis of GITB, one or more of these criteria had to be fulfilled, except for complete response to treatment. Complete resolution of symptoms and morphological (endoscopic and histological/microbiological) features after completion of standard ATT-$(HERZ)_2(HR)_{10}$ (with or without surgery) was taken as criteria for successful treatment.

Diagnosis of CD was based on morphological (radiological, endoscopic or surgical findings) and pathological criteria suggesting focal, asymmetrical, transmural or granulomatous features: (1) morphological: (a) discontinuous/segmental and asymmetrical mucosal involvement, (b) deep mucosal longitudinal fissures/ulcers, (c) transmural inflammation, (d) rigid and strictured intestinal wall, (e) presence of entero-cutaneous/entero-enteric fistula and/or chronic perianal disease; (2) pathological: (a) normal mucus content in the goblet cells of the inflamed region, (b) lymphocyte aggregation in the mucosa and submucosa, (c) non-caseating granuloma, (d) longitudinal ulcers/fissures, (e) transmural inflammation or inflammation beyond mucosa. For definite diagnosis of CD, the following criteria were used: presence of at least 3 different criteria or presence of non-caseating granuloma on histology with at least 1 other criterion; exclusion of TB (by histology, microbiological and PCR studies) and complete resolution of symptoms and morphological (endoscopic and histological/microbiological) features after 1-year treatment of corticosteroid and 5-ASA preparations (with or without surgery).

Study population

During the study period, definite diagnosis of CD was achieved in 26 patients. In analysing data retrospectively, all these patients with CD and an equal number of consecutive age- and sex-matched patients with definite diagnosis of GITB were included.

Statistical analysis

Data were analyzed using SPSS (PC+version-6 statistical package) by medical statistician. Univariate Chi-square test (UCST) was applied to find out statistically significant variables which may be important in differentiating CD from GITB. $P < 0.05$ was considered statistically significant. These variables were taken for multiple logistic regression (MLR) statistical analysis to find out independent predictive variables for diagnosis of CD. Each significant parameter (clinical, morphological, histological, microbiological and treatment-related) individually or in combination, was further analyzed for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy in diagnosis of CD or GITB.

RESULTS

Demographic, clinical and laboratory features of both the groups are demonstrated in Table 1. In CD and GITB groups, the following extra-intestinal features (%) were noted: (1) eye-related: episcleritis (3.8% and 0%), uveitis (0% and 0%); (2) skin and mucosa-related: erythema nodosum (7.7% and 3.8%), pyoderma gangrenosum (3.8% and 0%), vasculitis (7.7% and 0%), mouth ulcers (23.1% and 11.5%); (3) musculo-skeletal system related: ankylosing spondylitis (3.8% and 0%), arthritis (38.4% and 15.4%), osteopenia (15.4% and 0%); (4) hepatobiliary system-related: fatty liver (11.3% and 34.6%), autoimmune hepatitis (3.8% and 0%), primary sclerosing cholangitis (0% and 0%), gall stones (11.3% and 3.8%); (5) other system-related: thromboembolism (3.8% and 0%), renal stone (3.8% and 0%), amyloidosis (0% and 0%).

Table 1 Demographic, clinical and laboratory features

Characteristics	CD (n = 26)	GITB (n = 26)	P value
Demographic features			
Age (range), yr	36.6 ± 8.6 (6-79)	37.2 ± 9.6 (18-78)	NS
Sex (male:female) ratio	16:10	15:11	NS
Clinical features			
Duration of symptoms (range), mo	58.1 ± 9.8 (8-240)	7.2 ± 3.4 (2-24)	S
Chronic diarrhoea, *n* (%)	18 (69.2)	9 (34.6)	S
Hematochezia, *n* (%)	8 (30.7)	1 (3.8)	S
Fever, *n* (%)	6 (23.1)	18 (69.2)	S
Weight loss, *n* (%)	18 (69.2)	19 (73.1)	NS
Abdominal pain, *n* (%)	17 (65.4)	22 (84.6)	NS
Intestinal obstruction, *n* (%)	5 (19.2)	3 (11.5)	NS
Growth retardation, *n* (%)	2 (7.7)	0 (0)	NS
Ascites, *n* (%)	2 (7.7)	9 (34.6)	S
Abdominal lump, *n* (%)	2 (7.7)	4 (15.4)	NS
Extra-intestinal features, *n* (%)	16 (61.5)	6 (23.1)	S
Laboratory/radiological features			
Anaemia, *n* (%)	15 (57.7)	7 (26.9)	S
Hypoproteinemia, *n* (%)	11 (42.3)	9 (34.6)	NS
ESR (range) mm at 1 h	54.7 ± 12.2 (10-104)	59.7 ± 13.6 (19-110)	NS
Pulmonary infiltration/fibrosis, *n* (%)	1 (3.8)	10 (38.4)	S
Abdominal lymphadenopathy, *n* (%)	3 (11.5)	11 (42.3)	S
Generalized lymphadenopathy, *n* (%)	0 (0)	2 (7.7)	NS

Morphological features including site and type of lesions, histological and microbiological features as well as serological features are described in Table 2.

Among 26 patients with CD, 7 patients had at least one surgical intervention for intestinal complications before index presentation to our centre. They were not diagnosed as having Crohn's disease at that time, were not on therapy, presented at our centre with recurrence of symptoms, and were included for index evaluation and review of previous surgical histological specimens. Two patients had surgical intervention for intestinal stricture while on therapy for recurrent intestinal obstruction. All patients with CD showed complete response to therapy during the 1-year follow-up.

In GITB group, none of the patients had received ATT before the index evaluation. During the follow-up, 4 patients required surgical interventions for intestinal stricture while on therapy for recurrent intestinal obstructions. All the patients with GITB showed complete response to therapy during follow-up.

On UCST analysis, significant clinical variables were: (a) duration of symptoms, (b) diarrhoea, (c) hematochezia,

Table 2 Morphological, histological, microbiological, serological and treatment-related features *n* (%)

Characteristics	CD (*n* = 26)	GITB (*n* = 26)	*P* value
Sites of involvement			
Oesophagus	0 (0)	1 (3.8)	NS
Stomach	0 (0)	2 (7.7)	NS
Duodenum	1 (3.8)	2 (7.7)	NS
Jejunum	2 (7.7)	1 (3.8)	NS
Ileum	16 (61.5)	20 (76.9)	NS
Ileo-cecal valve	20 (76.9)	21 (80.7)	NS
Right colon	18 (69.2)	21 (80.7)	NS
Left colon	10 (38.4)	5 (19.2)	NS
Rectum	8 (30.7)	5 (19.2)	NS
Anal and perianal region	2 (7.7)	1 (3.8)	NS
Endoscopic and/or surgical findings			
Skip lesions	6 (23.1)	2 (7.7)	NS
Deep linear/serpiginous ulcers	15 (57.7)	6 (23.1)	S
Aphthous ulcers	9 (34.6)	5 (19.2)	NS
Stricture	8 (30.7)	5 (19.2)	NS
Cobble-stone appearance	15 (57.7)	6 (23.1)	S
Pseudo polyps	9 (34.6)	5 (19.2)	NS
Neoplasm-like appearance	0 (0)	2 (7.7)	NS
Fistula	3 (11.5)	0 (0)	NS
Histological features			
Granuloma	8 (30.7)	15 (57.7)	NS
Caseation	0 (0)	9 (34.6)	S
Confluence	0 (0)	6 (23.1)	S
Interpreted as IBD by pathologist	10 (38.4)	3 (11.5)	S
Interpreted as TB by pathologist	0 (0)	13 (50)	S
Interpreted as non-specific chronic inflammation by pathologist	16 (61.6)	10 (38.4)	NS
AFB smear/culture positivity	0 (0)	6 (23.1)	S
TB PCR positivity	0 (0)	17 (65.4)	S
Serological features			
p-ANCA	1 (3.8)	1 (3.8)	NS
c-ANCA	1 (3.8)	0 (0)	NS
IgA ASCA	10 (38.4)	6 (23.1)	NS
IgG ASCA	10 (38.4)	11 (42.3)	NS
Treatment-related factors			
Recurrence after surgery, *n*/total patients underwent surgery (%)	7/9 (77.7)	0/4 (0)	S
Number of patients previously treated with ATT (without any response)	9 (34.6)	-	-
Complete response to ATT	-	26 (100)	-

(d) fever, (e) ascites, (f) extra-intestinal manifestations. Variables found to be significant for differentiating CD and GITB based on MLR were duration of symptoms, diarrhoea, hematochezia and fever.

In Table 3, sensitivity, specificity, PPV, NPV and accuracy are tabulated for various parameters, which were significant on UCST, either individually or in combination. Clinical score (absence of fever, presence of diarrhoea and hematochezia and long duration of symptoms) had an accuracy of 84.62% to predict CD, with high (> 80%) sensitivity, specificity, PPV and NPV. Parameters like recurrence of disease after surgery, pathological diagnosis of TB, positive TB PCR and positive AFB smear/culture had 100% specificity and PPV, but had low sensitivity and NPV. The accuracy of various parameters in descending order were: clinical score (84.62%); positive TB PCR (82.6%); pathologic diagnosis of TB (75%); extra-intestinal focus of disease (pulmonary lesions and abdominal lymphadenopathy) (73.1%); morphology features (deep linear/serpiginous ulcers and cobble-stone appearance) (67.3%); pathologic diagnosis of IBD and granuloma (63.4%); positive AFB smear/culture (61.5%); and recurrence of disease after surgery (42.3%). Presence of caseating and/or confluent granuloma on histology, positive AFB smear and/or culture, positive TB PCR, histologic interpretation as TB, lack of response to ATT and recurrent disease after surgery were the only features which did not show any overlap between the two groups.

DISCUSSION

In spite of recent technical advances and better understating of pathophysiology, there is still no simple test for differentiating GITB from CD. There are many factors that lead to misdiagnosis. Both the diseases have marked overlap in demographic, clinical, radiological, endoscopic and surgical findings[10,22,23]. They can involve any part of gastrointestinal tract. Both are granulomatous diseases, so inability to find caseating or confluent granuloma on histology can lead to erroneous or non-confident diagnosis by a pathologist. Routine tests for diagnosis of TB such as AFB smear examination, using conventional Ziehl-Neelsen stain, traditional AFB culture using egg-based or agar-based media or Guinea pig inoculation, lack sensitivity and/or are time-consuming[29]. Smear examination using fluorescence technique and culture with Bactec technique are more rapid and more sensitive[29]. As GITB is a paucibacillary disease, sensitivity for detecting mycobacterium in clinical specimens by any of the above methods remains poor. Although many serological tests for TB are commercially available, they are far from satisfactory in diagnostic dilemma[29].

As we previously reported, features, which should alert a clinician for CD at presentation or on subsequent follow-up, were: (1) internal or cutaneous GI fistula; (2) perianal disease; (3) extra-intestinal manifestations, such as pyoderma gangrenosum, uveitis, primary sclerosing cholangitis, aphthous ulcers, arthralgia/arthritis or ankylosing spondylitis; (4) no caseating granuloma on histology and negative AFB smear or culture; (5) no involvement of the peritoneum, abdominal lymph node or site of extra-intestinal tuberculosis, such as extra-abdominal lymph node, lungs, pleura, mediastinum, liver, or kidney; and (6) no response to ATT[10].

In the present study, prolonged duration of illness, diarrhoea, hematochezia and extra-intestinal manifestations were more common in CD as compared to GITB; while fever and ascites were common i GITB. About an 85% accuracy in predicting CD could be reached based on the history of hematochezia, diarrhoea and protracted duration of symptoms, and absence of fever. Endoscopic features or surgical finding of deep linear/serpiginous ulcers/fissures and cobble-stone appearance was more common in CD. None of the clinical or morphological features was exclusive for either of the diagnoses.

Recently, Pulimood *et al* have reported that on mucosal biopsy, in addition to AFB detection, large granuloma, > four sites of granulomatous inflammation, caseation, band of epitheloid histiocytes in ulcer base and granulomatous inflammation in cecum in favour of diagnosis of TB; whereas non-caseating granuloma, mucosal changes distant

Table 3 Sensitivity, specificity and accuracy in diagnosis of CD or GITB

Parameters	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Factors predicting CD					
Clinical score (fever, hematochezia, diarrhoea and duration of symptoms)	80.7	88.4	87.5	82.1	84.62
Co-existing deep linear/ serpiginous ulcers and/or Cobble-stone appearance	69.2	65.3	66.6	72	67.3
Pathologist reporting as IBD	38.4	88.4	76.9	58.9	63.4
Recurrence of disease after surgery	77.7	100	100	66.6	42.3
Factors predicting GITB					
Co-existing pulmonary lesions and/or abdominal lymphadenopathy	57.6	88.4	83.3	67.6	73.1
Pathologist reporting as TB	50	100	100	66.6	75
Granuloma on histology	57.6	69.2	65.2	62	63.4
TB PCR positive	65.3	100	100	74.2	82.6
Smear and/or culture positive for AFB	23	100	100	56.5	61.5
Complete response to ATT	100	100	100	100	100

to sites with granuloma, focal crypt-related inflammation and granuloma in sigmoid or rectum in favour of diagnosis of CD[24]. In other studies, presence of small, discrete, loose mucosal granuloma without caseation are characteristics of CD, whereas large, dense, confluent granuloma with caseation with or without AFB positivity in mucosal or submucosal location favour TB[8,23,24,30]. In the present study, pathological diagnosis of TB had higher sensitivity, specificity and accuracy in predicting GITB than pathological diagnosis of IBD in predicting CD. Histological feature of caseation and/or confluence in granuloma and pathological diagnosis of TB were present in GITB group exclusively and there was no overlap in these findings.

Serological test for IBD is being investigated as diagnostic markers for IBD. Sensitivity of positive ASCA for diagnosis of CD reaches up to 76%, whereas it is 98% in various trials[27]. IgA ASCA and IgG ASCA are considered to be 95% specific for diagnosis of CD when individually tested, but 100% when tested in combination[26,27]. These data come from countries where tuberculosis is uncommon and control population in these studies did not include patients with GITB. In the previous study, we found that (1) serological markers were not significantly different between CD and GITB; and (2) prevalence of positive ASCA was much lower in patients with CD than the reported incidence from Western countries[28]. A recent study by another centre in India showed that ASCA was not helpful in differentiating CD from GITB[31]. The present study confirms these results.

TB PCR was found to be highly specific for GITB, but had poor sensitivity; these results were in accordance with our previous study[11]. TB PCR had accuracy of more than 80% in diagnosis of GITB. There was no false positive result in CD.

Since the sample size was small, patient groups were heterogenic (patients with involvement of both upper and/or lower GI tract were included) and conclusions are made based on prospective data analysis, a prospective study on all the patients with suspected CD or GITB with a larger sample size is needed to validate the clinical score.

In conclusion, simple clinical parameters like absence of fever, presence of hematochezia, presence of diarrhoea and long duration of symptoms have a highest pre-treatment accuracy in diagnosing Crohn's disease and differentiating it from intestinal tuberculosis. Microbiology and histology are useful adjuncts, whereas serology is not useful in differentiating intestinal tuberculosis from Crohn's disease.

COMMENTS

Background

Differentiating Crohn's disease (CD) from gastrointestinal tuberculosis (GITB) is a major problem in the tropical countries, as there is a marked overlap in clinical, radiological, endoscopic and surgical findings. In case of misdiagnosis as GITB, unnecessary anti-tuberculosis therapy (ATT) is given and treatment of CD is delayed. In contrast, in case of misdiagnosis as CD, treatment with steroids alone can lead to fatal dissemination of tuberculosis.

Research frontiers

This study prospectively evaluates various parameters for accuracy in diagnosis of CD or GITB.

Innovations and breakthroughs

Simple clinical parameters (duration of symptoms, fever, diarrhoea and hematochezia) turn out to have maximum accuracy in diagnosis of CD as compared with various laboratory, imaging, pathological, microbiological or serological features.

Applications

A prospective multi-center study involving a large sample size should be planned to validate results of this study.

Peer review

The authors present a nicely conceived and analyzed study with very good clinical data. This study investigates the distinguishing features of intestinal TB and CD. Obviously this is of great importance because of the divergence of management. While of immediate importance in India, it is also of interest because of the increase in CD with affluence in India and because this problem is occasionally seen in Western countries.

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RAPID COMMUNICATION

Role and outcome of conventional surgery in the treatment of pyogenic liver abscess in the modern era of minimally invasive therapy

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Abstract

AIM: To evaluate the role and outcome of conventional surgery in the treatment of pyogenic liver abscess in the modern era of minimally invasive therapy.

METHODS: The medical records of thirteen patients with pyogenic liver abscess who underwent surgical treatment between January 1995 and December 2002 were retrospectively reviewed to determine the clinical presentation, indication and nature of surgery, and outcome of surgery.

RESULTS: The patients were predominantly women (10/13) with a mean age of 65 ± 17 years. Their main presenting symptoms were abdominal pain (100%) and fever (77%). The aetiologies included biliary (*n* = 6), cryptogenic (*n* = 3), portal (*n* = 2), and trauma (*n* = 2). Seven patients underwent percutaneous drainage as the initial treatment. Of these, three patients developed peritonitis secondary to peritoneal spillage. Another four patients failed to respond because of multiloculation. Salvage surgery was required in these patients. Six patients proceeded to straight laparotomy: two had marked sepsis and multiloculated abscess that precluded percutaneous drainage, and four presented with peritonitis of uncertain pathology. Surgical procedures included deroofment and drainage (*n* = 9), liver resection (*n* = 3), peritoneal lavage (*n* = 2), cholecystectomy (*n* = 4), and exploration of common bile duct (*n* = 2). One patient required reoperation because of bleeding. Three patients required further percutaneous drainage after surgery. The overall mortality was 46%. Four patients died of multiorgan failure and two patients died of pulmonary embolism.

CONCLUSION: Surgical treatment of pyogenic liver abscess is occasionally needed when percutaneous drainage has failed due to various reasons. Mortality rate in this group of patients has remained high.

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Key words: Pyogenic liver abscess; Surgical drainage; Liver resection; Percutaneous drainage; Outcome

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INTRODUCTION

When not diagnosed early and treated promptly, pyogenic liver abscess can be fatal, with reported mortality rates as high as 80%-100%[1]. Historically, the treatment of choice for pyogenic liver abscess had been open surgical drainage[2]. However, with the advent of minimally invasive therapy such as image-guided percutaneous needle aspiration or catheter drainage and the availability of broad-spectrum antibiotics, patients with pyogenic liver abscess nowadays seldom require open surgery for treatment[3,4]. We aim to evaluate the role and outcome of conventional surgery in the treatment of pyogenic liver abscess in the modern era of minimally invasive therapy.

MATERIALS AND METHODS

Between January 1995 and December 2002, one hundred patients with pyogenic liver abscess were treated at our institution. Among them, thirteen patients required open surgical treatment. The medical records of these thirteen patients were retrospectively reviewed to determine the demographic data, clinical presentation, indications and nature of surgery, and outcome of surgery.

The diagnosis of pyogenic liver abscess was established by a combination of clinical, radiological, operative, and/or microbiological findings. All patients with suspected

Table 1 Clinical features, imaging results, preoperative diagnoses, and aetiologies of pyogenic liver abscess

No.	Sex/age	Coexisting diseases	Clinical features	US/CT findings	Preoperative diagnosis	Aetiology
1	F/74	History of jaundice	Peritonitis, hepatomegaly	18 cm left lobe cyst	Ruptured liver cyst	Biliary (GS)
2	F/83	HT, history of cholecystectomy	Pain, fever	2 cm left lobe abscess	Liver abscess	Biliary (CBDS)
3	M/66	DM	Pain, hepatomegaly	12 cm right lobe abscess	Liver abscess	Cryptogenic
4	F/83	HT, history of cholecystectomy and partial gastrectomy	Peritonitis, abdominal distension	(Dilated small bowel loops on abdominal X-ray)	Ischaemic bowel	Biliary (CBDS)
5	M/39	Recent blunt liver trauma with partial hepatectomy	Pain, fever	Haematoma and abscess along liver resection margin	Liver abscess	Trauma
6	M/34	Gunshot injury to liver with partial hepatectomy 4 months ago	Pain, fever, abscess pointing out	5.5 cm abscess communicating with anterior abdominal wound	Liver abscess	Trauma
7	F/86	HT, DM	Peritonitis, fever, hepatomegaly	Multiple liver cysts, largest one 18 cm in the right lobe	Ruptured liver cyst	Biliary (cystadenoma)
8	F/60	RPC, history of cholecystectomy and HJ	Pain, septic shock	5 cm left lobe abscess	Liver abscess	Biliary (RPC)
9	F/71	Diverticular disease	Pain, fever	13 cm right lobe abscess	Liver abscess	Portal (diverticulitis)
10	F/77	HT, history of thyroidectomy for cancer	Pain, fever	9 cm right lobe abscess	Liver abscess	Cryptogenic
11	F/60	No	Peritonitis, septic shock	Suspected acute cholecystitis	Acute cholecystitis	Biliary (GS)
12	F/64	No	Pain, septic shock	4.5 cm left lobe abscess	Liver abscess	Cryptogenic
13	F/47	DM	Pain, fever	9 cm left lobe and 3.5 cm right lobe abscesses	Liver abscess	Portal (diverticulitis)

HT: Hypertension; DM: Diabetes mellitus; RPC: Recurrent pyogenic cholangitis; HJ: Hepaticojejunostomy; GS: Gallstones; CBDS: Common bile duct stones.

liver abscess were initially investigated by ultrasonography (US) and/or computed tomography (CT) to confirm the diagnosis. Additional radiological studies including endoscopic or magnetic resonance cholangiography, and barium enema were subsequently performed in selected patients to look for the underlying aetiology. Pyogenic liver abscess was considered of cryptogenic origin when no obvious extra-hepatic source of infection could be identified after appropriate investigations. After the cultures of blood and/or liver pus aspirates (either by image-guided percutaneous aspiration or open surgery) were obtained, empirical intravenous antibiotics with ampicillin 500 mg six-hourly, cefuroxime 750 mg eight-hourly, and metronidazole 500 mg eight-hourly were administered. The antibiotics therapy was adjusted according to the results of culture and sensitivity test of pus aspirates. All patients received a six-week course of antibiotics.

The exact approach of initial intervention (percutaneous or surgical treatment) was determined by the patients' clinical condition, the certainty of diagnosis, the radiological findings, and the surgeons' preference. Percutaneous needle aspiration or catheter drainage was done under US-guidance, the technique of which had been reported previously[5]. Patients who failed percutaneous treatment (defined as deterioration of patients' clinical condition and/or persistence or progression of abscess on follow-up imaging studies) or developed procedure-related complications were subjected to surgery. For patients who underwent open surgical drainage, the abscess was located with the help of intraoperative US. Abscess wall was deroofed, and loculations were broken down. Large-bore catheters were placed within the abscess cavity for drainage. For patients with concurrent biliary pathologies, additional biliary tract procedures including cholecystectomy and exploration of common bile duct were performed. Liver resection was done for abscess with total destruction of a segment or section of liver parenchyma. Patients' outcomes, including length of hospital stay, recurrence of liver abscess on follow-up imaging, complications, need for re-intervention, and operative mortality were recorded.

RESULTS

Patients' demographic data and clinical presentation

The patients were predominantly women (ten out of thirteen) with a mean age of 65 ± 17 years. The main presenting symptoms were abdominal pain (100%) and fever (77%). Four patients presented with peritonitis of uncertain pathology despite radiological investigations. Three patients presented with septic shock requiring admission to the intensive care unit. Eleven patients (84.6%) had co-existing diseases (Table 1).

The aetiologies of the liver abscess included biliary (n = 6), cryptogenic (n = 3), portal (n = 2), and trauma (n = 2). The median abscess size was 9 cm (range, 2-18 cm). Eleven patients had a positive pus culture (with Klebsiella in eight patients), while a positive blood culture was only present in the three patients with septic shock (all grew Klebsiella).

Indications and nature of surgery

Seven patients underwent US-guided percutaneous drainage (PD) as the initial treatment. Of these, three patients (No. 2, 3, and 9) developed peritonitis secondary to peritoneal spillage of infected material. Another four patients (No. 5, 10, 12, and 13) failed to respond to PD because of multiloculation of the abscess. They all had persistent fever or progression of the abscess on follow-up US. Salvage surgical therapies, including deroofment and drainage, liver resection, peritoneal lavage, and additional biliary tract procedures, were required in these patients (Table 2). The median time interval between PD and salvage surgery was 3 d (range, 1-22 d).

Six patients proceeded to straight laparotomy. One

Table 2 Indications, nature, and outcome of surgery

	Initial intervention	Indications of surgery	No.	Details	Nature of surgery	Outcome
Pyogenic liver abscess (*n* = 13)	Ultrasonography-guided drainage attempted (*n* = 7)	Complications arising from percutaneous drainage (*n* = 3)	2	Peritoneal spillage	D&D	Survive
			3	Peritoneal spillage	Lavage	Survive
			9	Peritoneal spillage	Lavage	Survive
		Failed percutaneous drainage (*n* = 4)	5	Multiloculated abscess	D&D, debridement	Die of MOFS
			10	Multiloculated abscess	D&D, chole, ECBD	Die of PE
			12	Multiloculated abscess	L lat sec, chole	Die of MOFS
			13	Multiloculated abscess	L lat sec, D&D	Survive
	Straight laparotomy (*n* = 6)	Peritonitis of uncertain diagnosis (*n* = 4)	1	Ruptured liver cyst?	D&D, chole	Die of MOFS
			4	Ischaemic bowel?	D&D, ECBD	Die of MOFS
			7	Ruptured liver cyst?	D&D	Survive
			11	Acute cholecystitis?	D&D, chole	Die of PE
		Percutaneous drainage not suitable (*n* = 2)	6	Multiloculated abscess pointing out	D&D, debridement	Survive
			8	Multiloculated abscess in a septic patient	L lat sec	Survive

D&D: Deroofment and drainage; chole : cholecystectomy; ECBD: Exploration of common bile duct; L lat sec: Left lateral sectionectomy; MOFS: Multiorgan failure syndrome; PE: Pulmonary embolism.



Figure 1 Computed tomography of the abdomen showing liver disease. **A**: A liver abscess communicating with the anterior abdominal wound in a patient who had undergone partial hepatectomy for gunshot injury; **B**: A 5-cm liver abscess in the left lateral section with suspicious adjacent satellite abscesses in a patient who presented with septic shock; **C**: Multiple liver cysts in a patient who was subsequently proven to have an infected biliary cystadenoma that had ruptured.

patient (No. 6) had undergone partial hepatectomy for gunshot injury in China four months ago. He presented with a multiloculated liver abscess that was pointing out to the anterior abdominal surface (Figure 1A). Open drainage and debridement of necrotic liver tissues was performed. Another patient (No. 8) with underlying recurrent pyogenic cholangitis and previous history of hepaticojejunostomy presented with marked sepsis from a liver abscess in the left lateral section that precluded PD (Figure 1B). She underwent left lateral sectionectomy. The remaining four patients (No. 1, 4, 7, and 11) presented with peritonitis of uncertain pathology despite radiological investigations (Figure 1C). The preoperative diagnoses were ruptured liver cysts, ischaemic bowel, and acute cholecystitis. All four patients were proven to have ruptured liver abscess on laparotomy (Table 2).

Outcome of surgery

One patient (No. 1) required re-operation on day one because of the surgical complication of massive reactionary haemorrhage. Three patients required further PD after surgery because of intra-abdominal collection (No. 3 and 10) and the complication of biloma (No. 6). The overall mortality rate in this series was 46%. Four patients died of multiorgan failure and two patients died of pulmonary embolism (Table 2). For the seven patients who survived the surgery, the median hospital stay was 18 d (range, 14-27 d). None developed recurrence of liver abscess at a median follow-up of 63 mo (range, 10-108 mo).

DISCUSSION

Pyogenic liver abscess remains a rare but life-threatening disease with mortality rate as high as 80%-100% if untreated[1]. Historically, in the preantibiotic and reimaging era, the cornerstone of therapy for pyogenic liver abscess was open surgical drainage based on the classic work of Ochsner *et al* in 1938[2]. In 1953, McFadzean *et al* from Hong Kong first described the successful treatment of pyogenic liver abscess with closed aspiration and antibiotics[6]. With the introduction of high-resolution imaging modalities including US and CT in the recent two to three decades, image-guided PD has largely replaced surgical drainage as the mainstay of treatment[3,4]. This shift of practice has been guided by a drive for minimally invasive therapy whenever possible. PD has the advantages of avoiding general anaesthesia and an operative procedure, involves a shorter hospitalization, fewer complications, and better

patient acceptance[1]. Selected series of PD report success rates of more than 90%[5,7]. Surgical treatment has been reserved for patients who fail to respond to PD or who have concurrent intraabdominal pathology which requires surgical management[5,8].

In the present retrospective study, the surgical indications of the thirteen patients with pyogenic liver abscess include failure of PD, complications arising from PD, unsuitability of PD, and uncertain diagnosis. The reason for failure of PD in our patients is due to multiloculation of the abscess. Barakate *et al* have identified several predictors for failure of the percutaneous approach, including abscess multiloculation, presentation with abscess rupture, biliary communication, elevated serum urea, creatinine, and total bilirubin[8]. Multiloculation contributes to poorer drainage by compartmentalization of the abscess, which reduces the effectiveness of PD. Two septic patients in our study with multiloculated abscess and concurrent intraabdominal pathology (gunshot injury of the liver and recurrent pyogenic cholangitis) who were considered to be unsuitable for PD were subjected to straight surgery. Surgical drainage allows for breakdown of loculations and more complete drainage of large multiloculated abscess. In fact, some authors have advocated surgical drainage as the first-line treatment for septic patients with multiloculated abscess, as the perceived risk of failure of PD in this group of patients is high, and any delay in adequate drainage can lead to high mortality rate[9]. Another indication for primary surgical treatment, as shown in our study, is the presentation of peritonitis of uncertain diagnosis. All four patients were proven to have ruptured liver abscess on laparotomy. According to Barakate *et al*, abscess rupture is the single independent risk factor predicting failure of PD, and primary surgical treatment is strongly recommended for patients with abscess rupture on presentation[8].

Biliary tract diseases have become the most common aetiology of pyogenic liver abscess in the recent decades[3,4,7,8]. Although endoscopic retrograde cholangiopancreatography can easily deal with biliary obstruction due to common duct stones[10], other diseases like gallstones and recurrent pyogenic cholangitis with liver atrophy and intrahepatic duct stones will still require surgical treatment. Four patients in our study had undergone cholecystectomy for concurrent gallstone disease, while two patients had undergone exploration of common bile duct for clearance of common duct stones. Left lateral sectionectomy was performed in three patients with multiloculated abscess affecting segments Ⅱ and Ⅲ of the liver (one of them had underlying recurrent pyogenic cholangitis and intrahepatic duct stones and the other two had failed PD). Liver resection is particularly useful in patients with multiple abscesses or multiloculated abscess confined to a single lobe which cannot be drained even with open surgery[11].

With advancements in minimally invasive surgical techniques, laparoscopic management of pyogenic liver abscess has been shown by some authors to be feasible and safe[12-14]. The laparoscopic approach is a low-risk alternative to open surgery for patients who have failed PD[12]. As most patients requiring surgical intervention for pyogenic liver abscess are severely ill with sepsis, they may tolerate laparoscopic surgery better than open surgery. Comparing with open surgery, laparoscopic surgery has the advantages of faster recovery, less immunosuppression, shorter hospitalization, and better cosmesis[13]. Laparoscopy allows effective drainage of multiloculated abscess and thorough peritoneal lavage in case of ruptured abscess[14]. Laparoscopic US can also be done to localize concomitant abscesses and detect associated biliary pathology. In the hands of a skilled hepatobiliary and laparoscopic surgeon who is equipped with the proper tools, laparoscopic liver resection for liver abscess is also feasible[15].

The mortality rate for pyogenic liver abscess had been up to 40% until the 1980s[8]. Since then, more effective antibiotic therapy, improvement in imaging techniques, and development of image-guided PD, have resulted in published overall mortality rates between 6%-14%[5]. However, most reported series have included a heterogeneous group of patients who have undergone different treatment methods, including medical treatment alone, PD, and surgery. For the subgroup of patients undergoing surgical treatment, the mortality rate still averages 20%[16-19]. The overall mortality rate in the present surgical series is 46%. Four patients died of multiorgan failure and two patients died of pulmonary embolism. In a multivariate analysis of forty-six patients with pyogenic liver abscess, Mischinger *et al* found that a high Acute Physiology and Chronic Health Evaluation (APACHE) Ⅱ score and the underlying disease (malignancy) but not the mode of therapy was independent predictors of mortality[18]. Chou *et al* also found that the systemic effects of liver abscess with sepsis and multiple organ failure were significant factors in predicting mortality[19]. In the present study, no statistical analysis of prognostic factors is performed because of the small sample size. However, we believe that the high mortality rate may be attributed to the large size of the liver abscess (median size 9 cm), a higher proportion of patients with abscess multiloculation and abscess rupture on presentation, and the associated comorbidities of the patients. Moreover, the highest mortality is observed in the subgroup of patients who failed PD and underwent salvage surgery (three out of four died). The median time interval between PD and salvage surgery was 3 d. According to Herman *et al*, in a septic patient with pyogenic liver abscess, aggressive therapy should be instituted as soon as possible, as any delay or failure of adequate drainage would increase morbidity and mortality[9]. For this reason, they recommended surgical drainage instead of PD as first-line treatment in septic patients, and they were able to show a low mortality of 8.5% in their series. Recently, a low mortality rate of 4.5% was also reported by Tan *et al*, who subjected patients with pyogenic liver abscess larger than 5 cm in size to surgical drainage as first-line treatment[20].

In conclusion, because of its minimally invasive nature, PD is still regarded as the first-line treatment for most patients with pyogenic liver abscess. Surgical treatment is occasionally required when PD has failed, when the patients have concurrent intraabdominal pathology, or when the diagnosis is uncertain. In selected group of septic patients with large and multiloculated abscess, surgical drainage may be used as the first-line treatment. However, the overall mortality rate in patients undergoing surgical treatment has remained high.

COMMENTS

Background

Pyogenic liver abscess remains a rare but life-threatening disease with mortality rate as high as 80%-100% if untreated. Historically, the treatment of choice for pyogenic liver abscess had been open surgical drainage. However, with the advent of minimally invasive therapy such as image-guided percutaneous needle aspiration or catheter drainage and the availability of broad-spectrum antibiotics, patients with pyogenic liver abscess nowadays seldom require open surgery for treatment. The aim of this study was to evaluate the role and outcome of conventional surgery in the treatment of pyogenic liver abscess in the modern era of minimally invasive therapy.

Research frontiers

It is still uncertain whether first-line treatment with surgical or percutaneous drainage of large pyogenic liver abscess (size > 5 cm) results in better clinical outcome. This controversial issue can only be properly addressed by a randomised controlled trial. With advancements in minimally invasive surgical techniques, laparoscopic management of pyogenic liver abscess has been shown by some authors to be feasible and safe. Further studies to compare the efficacy and safety of laparoscopic versus open drainage of pyogenic liver abscess should be conducted.

Innovations and breakthroughs

This study does not intend to bring about any major innovations or breakthroughs in the management of pyogenic liver abscess. It mainly serves as a reminder to readers of the modern era treatment planning for pyogenic liver abscess, with a special emphasis on the role and outcome of conventional surgery in the treatment of this old but deadly pathology.

Applications

This study serves as a reminder to readers of the management algorithm of pyogenic liver abscess in the modern era of minimally invasive therapy. Because of its minimally invasive nature, percutaneous drainage is still regarded as the first-line treatment for most patients with pyogenic liver abscess. Surgical treatment is occasionally required when percutaneous drainage has failed, when the patients have concurrent intraabdominal pathology, or when the diagnosis is uncertain. In selected group of septic patients with large and multiloculated abscess, surgical drainage may be used as the first-line treatment.

Terminology

'Pyogenic liver abscess' is a pus-filled lesion within the liver caused by bacterial infection. 'Percutaneous drainage' is a minimally invasive treatment option for pyogenic liver abscess, which can be done under local anaesthesia. Under X-ray or ultrasonography guidance, a fine catheter is inserted through the skin (*via* a tiny skin incision) into the liver abscess cavity for pus drainage.

Peer review

This study serves as a reminder to readers of the modern era treatment planning for an old deadly pathology. Patients with pyogenic liver abscess are at very high risk of decompensation with rapid progression to multi-system failure and they need to be sent to appropriate centres for expedited appropriate stepwise treatment.

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RAPID COMMUNICATION

Reconstruction of the abdominal wall by using a combination of the human acellular dermal matrix implant and an interpositional omentum flap after extensive tumor resection in patients with abdominal wall neoplasm: A preliminary result

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Abstract

AIM: To present our trial using a combination of the human acellular dermal matrix (HADM) implant and an interpositional omentum flap to repair giant abdominal wall defects after extensive tumor resection.

METHODS: Between February and October of 2007, three patients with giant defects of the abdominal wall after extensive tumor resection underwent reconstruction with a combination of HADM and omentum flap. Postoperative morbidities and signs of herniation were monitored.

RESULTS: The abdominal wall reconstruction was successful in these three patients, there was no severe morbidity and no signs of herniation in the follow-up period.

CONCLUSION: The combination of HADM and omentum flap offers a new, safe and effective alternative to traditional forms in the repair of giant abdominal wall defects. Further analysis of the long-term outcome and more cases are needed to assess the reliability of this technique.

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Key words: Abdominal wall neoplasm; Abdominal wall reconstruction; Human acellular dermal matrix; Omentum flap

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INTRODUCTION

The treatment of abdominal wall neoplasms, whether primary or secondary, continues to present a challenging problem to general surgeons. Extensive tumor resection of those neoplasms can result in giant abdominal wall defects. Reconstruction of the abdominal wall is still not easy to an average general surgeon, even to a plastic surgeon. Most reports described reconstructing giant abdominal wall defects by using synthetic mesh implants, autologous tissue graft, or a combination of the two methods mentioned above.

Synthetic mesh such as polypropylene (PP) and expanded polytetrafluoroethylene (ePTFE) are the mainstays of ventral hernia repair and commonly used to repair other trunk defects today. For PP, as a result of its macroporous structure, the mesh induces intense fibrovascular infiltration and incorporates into the surrounding musculofascial tissue to provide a strong repair. However, it is also associated with adhesions to intraabdominal viscera and enterocutaneous fistula formation[1-3]. ePTFE has been used in an attempt to reduce these complications and has been reported to cause fewer adhesions to the bowel because of its microporous surface structure. However, incorporation into the surrounding tissue is limited[4-6]. Furthermore, both of them contain permanent prosthetic material and are largely intolerant of infection[7-10], particularly if used in contaminated fields or in the event of exposure owing to dehiscence or breakdown of the overlying skin.

However, abdominal wall tumors, particularly secondary ones, are frequently accompanied by contamination or infection. Such situations are a contradiction for implanting PP or ePTFE. Absorbable mesh, such as Vicryl can be used as a temporary support in contamination or infection situation[11,12]. But the patient needs a second-stage operation for implanting an inabsorbable mesh to complete a definite repair. Additionally, some secondary tumors invade the complete abdominal wall including skin. Therefore, a single mesh is not enough without skin coverage.

The use of autologous tissue flaps, in most time musculocutaneous flaps such as tensor fasciae latae, rectus femoris, rectus abdominis and latissimus dorsi, combined with or without incorporation of synthetic meshes, has been described in the successful repair of abdominal wall defects[13-16]. It eliminates the complications associated with synthetic implant intolerance and is a good replacement of the full-thickness abdominal wall. Despite the advantages of autologous tissue, the creation of a donor site, the potential for donor site morbidity and limited size decrease this technique's application[17,18]. The preparation of a musculocutaneous flap, whatever pedicled or free, greatly increases operation time, complexity, and are generally outside the scope of practice of many general surgeons[13,19].

Recently, some biologic materials were introduced to hernia repair and abdominal wall reconstruction. Human acellular dermal matrix (HADM) is a biologic material derived from human cadaveric dermis. In 1992 it was first used as a dermal replacement in burn surgery[20]. Since then it has been used successfully in the repair of oral defects and replacement of soft tissues in plastic and pelvic procedures[21-25]. The advantages of HADM is its resistance to infection, the absence of a permanent foreign body at the repair site and its excellent mechanical properties (strength to failure, plasticity and flexibility)[23,26-28]. These properties make it an attractive alternative to prosthetic mesh in abdominal wall replacement. Animal models and some case reports using HADM have shown successful hernia repair, abdominal wall reconstruction and resistance to infection in contaminated cases[1,29-39]. Therefore, HADM is an ideal alternative to synthetic mesh for abdominal wall restoration after tumor resection, especially in the situation of infection or contamination.

The omentum serves a multipurpose in the medical field. The pedicled omentum flap is a special autologous tissue flap, but its preparation is much easier than musculocutaneous flaps. It has been reported to reconstruct abdominal wall defects successfully[40-43]. The omentum consists of abundant blood vessels, fat, and lymphatic tissue and is known for its unique immunologic and angiogenic properties[44]. We suppose, in the reconstruction of abdominal wall after tumor resection, it is a good alternative to the musculocutaneous flap and favorable for HADM remodeling after its implantation.

The purpose of this article is to present our approach of using a combination of the HADM implant and an interpositional omentum flap to repair giant abdominal wall defects after extensive tumor resection.

MATERIALS AND METHODS

Patients

Between February and October of 2007, three patients (two men and one woman) with giant defects of the abdominal wall after extensive secondary tumor resection underwent reconstruction with HADM (8 mm thickness, Renov, China) and omentum flap. The first patient was a 51-year-old woman with secondary gallbladder carcinoma, the second one was a 66-year-old man with secondary renal carcinosarcoma, and the third was a 62-year-old man with secondary sigmoid carcinoma. Defect size ranged from 10 cm × 8 cm to 22 cm × 16 cm. The first patient had an overlying skin defect that was covered with a free thigh skin graft and the later two had enough skin and subcutaneous tissues coverage.

Surgical technique

Operations are performed by a team consisting of general and plastic surgeons. Initially, the tumor was widely resected at least 3-5 cm beyond the margin of the tumor and completeness of clearance was ascertained by frozen sections. The abdominal cavity was entered and any bowel that was adherent to the parietal peritoneum along the fascial margin was freed to create space for the underlying portion of the HADM patch.

Then, the greater omentum was evaluated with regard to its size, vascular pattern, and reach for a given defect. The omentum was mobilized fully and pedicalized on either the right or left gastroepiploic arteries. The exit point was a 3-4 cm slit, located on the side ipsilateral to the defect. The right subcostal site is preferred because the liver protects against herniation of bowel.

Next, the HADM patch was applied using an underlay technique. The number of HADM sheets used depended on the size of the defect. After rehydration, several sheets of HADM were sewn together and inserted as an underlay patch with interrupted horizontal mattress sutures. An allowance of 2-3 cm circumferentially was given for the underlaid portion. The patch should be taut but not excessively tight to achieve a tension-free repair. The omentum flap was then gently spread over the HADM and loosely interrupted sutured to the muscle and fascia margin with 0 Prolene. Closed suction drains were placed above and below the omentum flap.

Finally the subcutaneous tissues and skin or graft skin was closed above the omentum. All patients were given abdominal binders postoperatively for additional support for at least 6 mo while the repair matured.

RESULTS

Case 1

A 51-year-old woman presented with secondary gallbladder carcinoma over her upper abdominal wall. She had a history of radical operation of gallbladder carcinoma three years before and was diagnosed with abdominal wall metastasis two years postoperation. Radiotherapy and systemic chemotherapy proved not to be effective and an ulcer with infection developed one year later (Figure 1A).



Figure 1 Case 1. **A**: The view of the abdominal wall before operation; **B**: The defect after extensive tumor resection; **C**: Reconstruction with HADM which was placed in an underlay fashion. The pedicled omentum flap was brought into the subcutaneous plane by means of a 4-cm slit at the left subcostal site; **D**: The HADM patch was completely covered by the omentum flap; **E**: A left free thigh skin graft was raised to cover the skin defect; **F**: The view of abdominal wall 1 mo postoperatively.

Intraoperatively, the tumor was noted to involve the entire thickness of the abdominal wall and adhered to the left lobe of the liver and pylorus. Wide excision resulted in a full-thickness upper abdominal wall defect measuring 22 cm ×16 cm (Figure 1B). The greater omentum was mobilized fully and pedicalized on the left gastroepiploic artery. The exit point was a 4 cm slit, located on the left subcostal site. Reconstruction was performed using the HADM patch described above. Five sheets of HADM (four 10 cm × 8 cm and one 10 cm × 5.5 cm) were sewn together and inserted as an underlay patch with 0 Prolene interrupted horizontal mattress sutures (Figure 1C and D). The overlying skin defect was covered with a free thigh skin graft (Figure 1E). She recovered uneventfully (Figure 1F). At 7-mo follow-up, the repair was noted to be intact and there was no sign of herniation. Several metastatic lesions were found in her left lung at the sixth month post-operation, but the patient is still alive.

Case 2

A 66-year-old man presented with recurrent renal carcinosarcoma over his right upper lateral abdominal wall (Figure 2A). He had a history of renal carcinosarcoma excised half a year previously. Intraoperatively, the tumor was found between the peritoneal membrane and muscle. There was no invasion of the subcutaneous tissue. Wide excision resulted in a right lateral abdominal wall defect measuring 15 cm × 11 cm (Figure 2B), but the excision was not complete in the original renal zone because some tumor tissue was located beside the spinal column and circled around the inferior caval vein which was only leaving for radiotherapy and chemotherapy. Reconstruction was performed as similar as in case 1. One sheet of 10 cm × 8 cm and one of 10 cm × 5.5 cm HADM were sewn together and inserted as an underlay patch. The upper part was fixed on the diaphragm. The omentum was transposed extraperitoneally through a 4-cm opening on the right subcostal site (Figure 2C and D). The subcutaneous tissue and skin were closed above the omentum flap (Figure 2E). He recovered uneventfully (Figure 2F). At 4-mo follow-up, the reconstruction remains stable and he is able to perform normal daily activities.

Case 3

A 62-year-old man presented with an advanced sigmoid colon carcinoma, which perforated chronically into the lower part of the left musculus rectus abdominis and infiltrated to the bladder. Computed tomography demonstrated infiltration through the peritoneum to the abdominal wall muscles. Excision of the abdominal lesion was performed with a 2 cm lateral margin, removing the parietal peritoneum and the full thickness of the muscle. Intraoperative frozen section revealed clear resection margins. This resulted in a defect measuring 10 cm × 8 cm. Reconstruction was performed similar to case 2. The omentum was mobilized and pedicled on the left gastroepiploic artery. The exit point was located on the inner side of the defect. The patient's post-operative recovery was uneventful and there was no infection of his abdominal wall. The patient was followed up for only 1 mo and there is no herniation up to now.

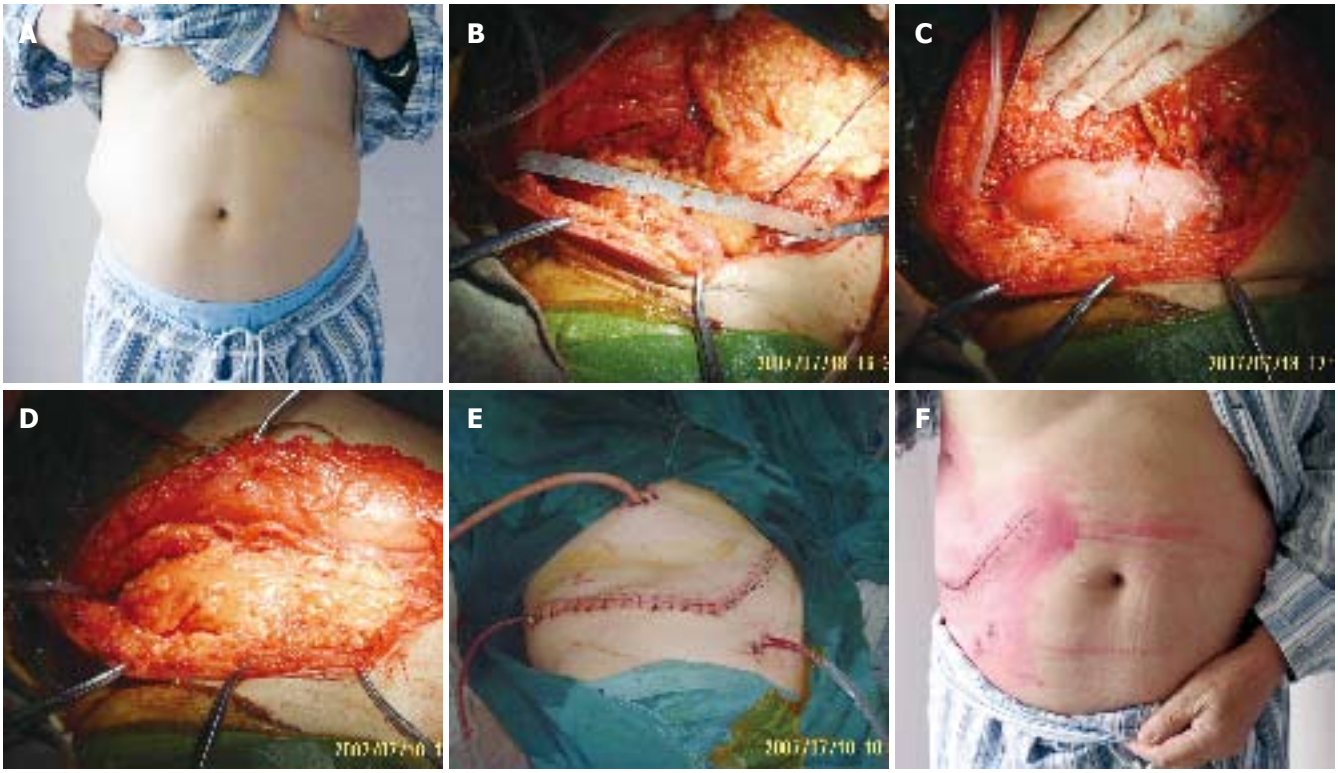


Figure 2 Case 2. **A**: The view of the abdominal wall before operation; **B**: The defect after extensive tumor resection; **C**: Reconstruction with HADM which was placed in an underlay fashion. The pedicled omentum flap was brought into the subcutaneous plane by means of a 4-cm slit at the right subcostal site; **D**: The HADM patch was completely covered by the omentum flap; **E**: The subcutaneous tissue and skin were closed above the omentum flap; **F**: The view of the abdominal wall 2 wk after operation.

DISCUSSION

Extensive resection of abdominal wall tumors frequently leads to a giant defect. Additionally accompanying with infection or contamination, repairing such a giant defect is still a challenging task to general surgeons. The current techniques of abdominal wall reconstruction all have their disadvantages: the transferring of a musculocutaneous flap creates a donor site where it can create potential donor site morbidities and is time-consuming. Inabsorbable synthetic mesh is intolerant to infection and sometimes results in an excessive foreign body reaction, absorbable mesh is sometimes used only as a temporary support of abdominal wall in a two stage operation. Therefore, new alternatives are called on to replace the old techniques. Here, we report our trial in three secondary abdominal wall tumor patients by combining HADM with an interpositional omentum flap as a technique to successfully repair giant abdominal wall defects after extensive tumor resection.

HADM is a biologic material derived from human cadaveric dermis. Chemical and physical processing removes all cellular components of the dermis while preserving the extracellular matrix and basement membrane components. This results in a sheet consisting of extracellular material that acts as a signal for fibroblast incorporation, collagen deposition, and collagen maturation. These changes occur when the material is implanted into the human body[26-28]. The extracellular matrix stays intact and is gradually revascularized and remodeled into autologous tissue while maintaining its structural integrity. Animal models proved that early revascularization of the graft is thought to enhance resistance to infection and contamination[29]. So its biggest benefit is its tolerance of overlying infection without breakdown or loss of strength. Additionally, the advantages of HADM also include the absence of a permanent foreign body at the repair site and excellent mechanical properties, such as strength to failure, plasticity and flexibility[23,26-28]. Based on these facts, we believed that HADM is an ideal alternative of synthetic mesh for abdominal wall restoration after tumor resection, especially in the infected or contaminated situation.

A pedicled omentum flap is easy to prepare, and in most times it can reach defects over all quadrants of the abdominal wall. The omentum, consisting of abundant blood vessels, fat, and lymphatic tissue, is known for its unique immunologic and angiogenic properties. Therefore, it can be used in infected or contaminated situations. By using it as an interpositional flap, we harness such properties to facilitate wound healing[44-45] and promote remodeling of HADM after implantation. We have demonstrated that the omentum, with its size, vascularity and bulk, can be used successfully as a local flap in combination with the HADM in abdominal wall restoration. Although an omentum flap is not stronger than a musculocutaneous flap, the HADM sheet plays a role of resisting pressure from the abdominal cavity, and an additional free skin graft could easily be taken for the patient who has a full-thickness abdominal wall defect. An omentum flap is somewhat easier to prepare compared to a musculocutaneous flap which avoids a donor site and its associated morbidities and additionally shortens the operation time. Sometimes it also avoids microsurgery that is needed for a free musculocutaneous flap, which

represents an advantage in patients with significant comorbidities such as diabetes. In our cases, an omentum flap combined with HADM allowed us to repair a giant abdominal wall defect after an extensive tumor resection in a relatively short operation with no severe morbidity. To our knowledge, this technique has never been described before.

Up to now, no herniation was observed in these three patients. But we know that the number of our cases is too small and the follow-up period is too short. Because of the theorized structural remodeling of HADM, the long-term integrity of the abdominal wall after reconstruction remains in question. But this technique may potentially be used in the restoration of abdominal wall after extensive tumor resection, especially in patients presenting with a secondary tumor with infection or contamination.

In conclusion, the combination of HADM and omentum flap offers a safe and effective alternative to traditional forms in the repair of giant abdominal wall defects. The HADM appears to have the ability to survive in contaminated or infected surgical fields, the omentum flap is easy to prepare and avoids a donor site defects and morbidities that may result from a free or pedicled musculocutaneous flap transferring. The combination offers the opportunity for a single-staged reconstruction after extensive tumor resection. That also means it provides an option when neither synthetic prosthetic mesh nor musculocutaneous flap can be used to achieve closure. Further analysis of the long-term outcome and more cases also are needed to assess the reliability of this technique; nonetheless favorable short-term results are a welcome change for the surgeon called on to deal with such challenging dilemma.

COMMENTS

Background

Extensive resection of abdominal wall tumor frequently leads to a giant defect, which is difficult to repair for general surgeons. The current techniques of abdominal wall reconstruction, such as transferring a musculocutaneous flap and implanting a synthetic mesh have their disadvantages. Here, we report our trial in three secondary abdominal wall tumor patients by combining human acellular dermal matrix (HADM) with an interpositional omentum flap as a technique to successfully repair giant abdominal wall defects after extensive tumor resection.

Innovations and breakthroughs

The technique of combining the HADM implant and an interpositional omentum flap is to be used for the first time to repair giant abdominal wall defects after extensive tumor resection.

Applications

The combination of HADM and omentum flap offers a safe and effective alternative to traditional forms in the repair of giant abdominal wall defects after extensive tumor resection, especially in secondary tumor patients with infection or contamination.

Terminology

HADM: a biologic material derived from human cadaveric dermis, which has been used successfully in the repair of oral, abdominal and other tissue defects. Pedicled omentum flap: a special autologous pedicled flap which serves multipurpose in medical field.

Peer review

This manuscript presents a new way to reconstruct giant abdominal wall defects using the combination of a HADM implant and an interpositional omentum flap which appears to provide a safe and reliable alternative in this area.

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RAPID COMMUNICATION

LINE-1 family member *GCRG123* gene is up-regulated in human gastric signet-ring cell carcinoma

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Abstract

AIM: To analyze the expression profiles of a human gastric-cancer-related gene, *GCRG123*, in human gastric signet-ring cell carcinoma tissues, and to perform bioinformatics analysis on *GCRG123*.

METHODS: *In situ* hybridization was used to explore the *GCRG123* expression pattern in paraffin-embedded gastric tissues, including 15 cases of signet-ring cell carcinoma, 15 of intestinal-type adenocarcinoma, and 15 of normal gastric mucosa. Northern blotting was used to analyze the differences in *GCRG123* expression between stomach signet-ring cell carcinoma and intestinal-type adenocarcinoma tissues. Online software, including BLAST, Multalin and BLAT, were applied for bioinformatics analysis. National Center for Biotechnology Information (NCBI) and the University of California Santa Cruz (UCSC) databases were used for the analyses.

RESULTS: The *in situ* hybridization signal appeared as blue precipitates restricted to the cytoplasm. Ten out of 15 cases of gastric signet ring cell carcinoma, normal gastric mucosal epithelium and pyloric glands showed high *GCRG123* expression. Low *GCRG123* expression was observed in gastric intestinal-type adenocarcinoma and normal gastric glands. Northern blotting revealed that *GCRG123* was up-regulated in signet-ring cell carcinoma tissue but down-regulated in intestinal-type adenocarcinoma tissue. BLAST and Multalin analyses revealed that the *GCRG123* sequence had 92% similarity with the ORF2 sequence of human long interspersed nuclear element retrotransposons (LINE-1, L1). BLAT analysis indicated that *GCRG123* mapped to all chromosomes. *GCRG123* was found to integrate in the intron-17 and -23 of Rb, 5′ flanking region of IL-2 and clotting factor IX genes.

CONCLUSION: *GCRG123*, an active member of the L1 family, was up-regulated in human gastric signet-ring cell carcinoma.

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Key words: Stomach; Signet-ring cell carcinoma; *GCRG123*; LINE-1; *In situ* hybridization; Bioinformatics

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INTRODUCTION

Gastric cancer remains one of the most common forms of cancer worldwide with approximately 870 000 new cases and 650 000 deaths per year[1-4], which accounts for about 9.9% of new cancers[5]. Worldwide, there has been a decline in the incidence of the intestinal type gastric cancer in the last few decades, following the overall decline in the incidence of gastric cancer. By contrast, the decline in the diffuse type gastric cancer has been more gradual. Some studies have reported an increase in the diffuse type of gastric carcinoma, especially the signet-ring cell type[6]. As a result, the diffuse type now accounts for about 30% of gastric carcinomas in some reported series[7]. Intestinal-type and diffuse-type gastric cancer differ in their epidemiology, pathogenesis, genetic profile, and clinical outcome[8].

In 1999, we cloned a gene segment *GCRG123*, which was down-regulated in gastric adenocarcinoma. BLASTX analysis revealed that the predicted *GCRG123* product was a lamin-like protein[9]. Recently, we have used the updated GeneBank database for further analysis on the *GCRG123* sequence. We found that *GCRG123* appeared to be a long interspersed nucleotide element-1 (LINE-1, L1) family member. Additionally, we revealed that *GCRG123* was ultimately up-regulated in stomach signet-ring cell carcinoma, as well as in normal pyloric glands and epithelia, which shows an opposite expression pattern

compared with its down-regulation in stomach intestinal-type adenocarcinoma.

MATERIALS AND METHODS

Patients and tissue acquisition

All samples were obtained from the Department of Pathology, General Hospital of Chinese PLA. Specimens of paraffin-embedded gastric tissues, including 15 cases of gastric signet-ring cell carcinoma, 15 of advanced gastric intestinal-type adenocarcinoma and 15 of normal gastric mucosal tissues, were collected for *in situ* hybridization analysis. One set of fresh gastric signet-ring cell carcinomas and paired non-cancerous gastric tissues, and one set of fresh gastric intestinal-type adenocarcinomas and paired non-cancerous gastric tissues were used for Northern blot analysis. The diagnosis of cancer was confirmed through histology.

In situ hybridization

cRNA probe labeling: Digoxigenin-labeled anti-sense and sense cRNA probes were prepared by *in vitro* transcription (DIG RNA Labeling Kit (SP6/T7); Roche Diagnostics, Mannheim, Germany). Briefly, the following labeling procedure was employed: purified *GCRG123* cDNA 100 ng/10 μL, 5 × NTP labeling mixture 4 μL, 5 × transcription buffer 4 μL, and RNA polymerase SP6/T7 2 μL were mixed gently, centrifuged, and then incubated for 1 h at 42℃. Two microliters of RNase-free DNase I was added to remove template DNA, by incubating for 15 min at 37℃, and the reaction was finally stopped by adding 2 μL 0.2 mol/L EDTA (pH 8.0). Labeling efficiency was directly detected by a spot test as described in the protocol of the kit.

Hybridization: All specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. A series of 5-μm thick sections were cut for analysis. *In situ* hybridization was performed as previously described[10,11] with some amendments, using digoxigenin-labeled anti-sense cRNA probes. Briefly, the slides were dried at 40℃ overnight, dewaxed, rehydrated and pretreated with DEPC-treated PBS containing 100 mmol/L glycine and 0.3% Triton X-100, respectively. The sections were then permeabilized with 20 μg/mL RNase-free proteinase K (Merck, Darmstadt, Germany) for 20 min, incubated at 37℃ for at least 20 min with prehybridization buffer. Each section was overlaid with 30 μL hybridization buffer containing 10 ng digoxigenin-labeled cRNA probe and incubated at 42℃ overnight. After hybridization, the section was incubated with digoxigenin antibody (75 mU/mL) for 2 h. The positive signal for *GCRG123* mRNA was detected by using NBT/BCIP (Promega, WI, USA) as a substrate. Sense cRNA probes were used as a negative control. The presentation of blue staining in the cytoplasm was considered positive. The positive staining of cytoplasm was scored manually as described below: -, barely detectable light blue; 1+, diffuse light blue; 2+, blue; 3+, dark blue. More than 100 non-tumor or tumor cells were quantified in each measurement, and more than one measurement was required to confirm the diagnosis. Consequent slides with H&E staining were then reviewed to compare the histological patterns to the staining patterns in the *in situ* hybridization preparations.

Northern blot analysis

Dig Northern Starter Kit (Roche Diagnostic, Indianapolis, IN, USA) was used. The procedure of hybridization was performed according to the manufacturer's protocol. Digoxigenin-labeled cRNA probe was generated as described above. Digoxigenin-labeled sense cRNA probe was used as a negative control. The hybridization signals were visualized with chemiluminescence, which was recorded on X-ray films. The exposure time was 10 min.

Bioinformatic analysis

BLAST (http://www.ncbi.nlm.nih.gov/BLAST/)[12] was used for sequence similarity search. MultAlin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html)[13] was used for multiple sequence alignment analysis. BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat) was applied to find the genomic coordinates of mRNA, and to search for gene family members[14]. Alignment parameters were set as default. NCBI and UCSC databases were used for the analyses.

Informed consent

The study protocol was approved by the Institutional Review Board of the hospital under the guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all patients.

RESULTS

In situ hybridization

GCRG123 expression was analyzed at the mRNA level using *in situ* hybridization. The hybridization signal appeared as blue staining in the cytoplasm. Fourteen gastric intestinal-type adenocarcinoma cases showed - to ± staining (Figure 1A). Interestingly, 10 out of 15 gastric signet-ring cell carcinoma tissues were stained 2+ to 3+ (Figure 1B), while the other signet-ring cell tissues were stained ± to +. In the normal gastric mucosa, all gastric epithelia and pyloric glands were heavily stained (grade 2+ to 3+) (Figure 1C and D), however, the normal gastric glands were stained lightly (Figure 1C).

Northern blotting

Both normal gastric tissue and signet-ring cell carcinoma tissue showed strong hybridization signals, while the gastric intestinal-type carcinoma tissue showed a weak signal. These results seem to indicate that *GCRG123* expression was higher in gastric signet-ring cell carcinoma than in intestinal-type adenocarcinoma (Figure 2).

Bioinformatic analysis

The sequence of *GCRG123* was enrolled for a sequence-similarity search in the GenBank database. *GCRG123* shared 92% similarity with the human transposon L1 (M80343) sequence, as well as the highly active L1 sequence reported

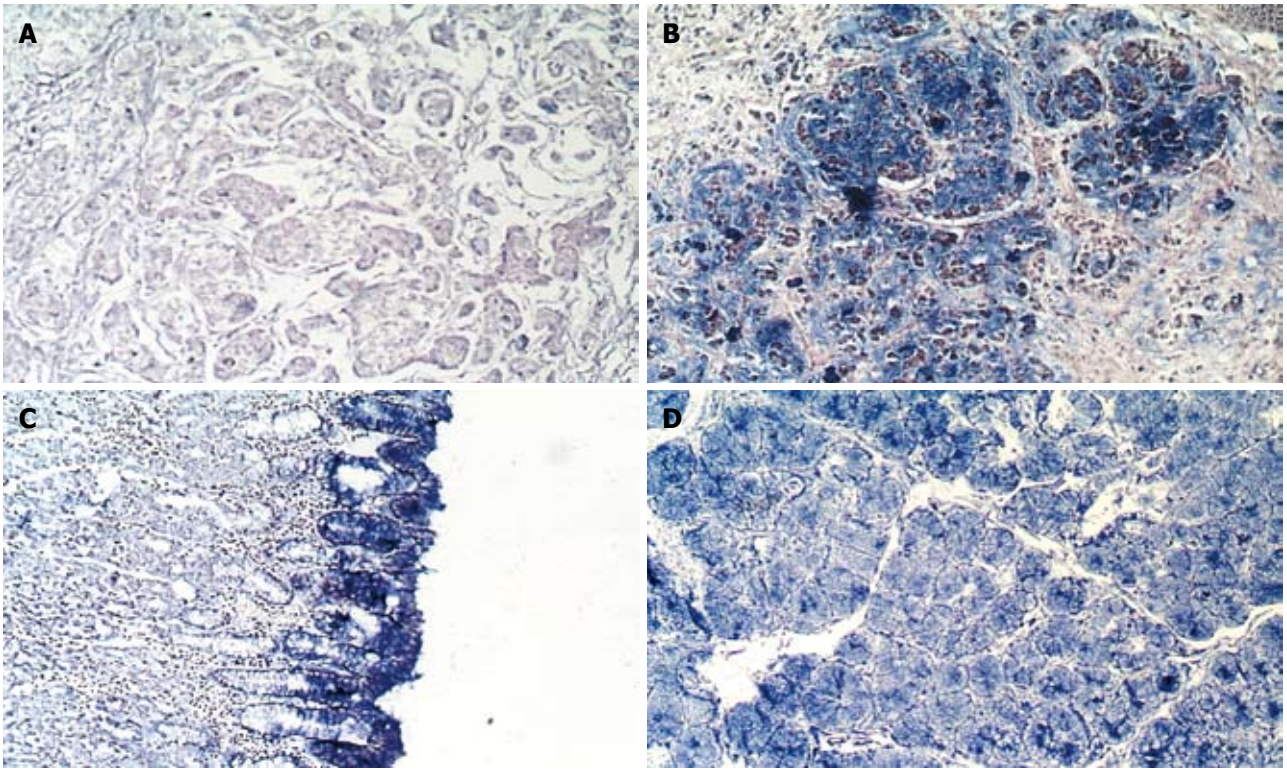


Figure 1 *In situ* hybridization analysis of *GCRG123* in gastric mucosa. *GCRG123* showed low expression (blue precipitates restricted to the cytoplasm) in intestinal-type adenocarcinoma (**A**) and normal gastric glands (**C**, left region); high expression in signet-ring cell carcinoma (**B**); normal epithelia (**C**); and normal pyloric glands (**D**). NBT/BCIP was used as an alkaline phosphatase substrate (magnification was 10 × 10).

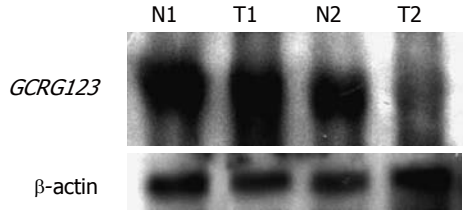


Figure 2 Northern blotting of *GCRG123* in gastric tissues. The expression of β-actin served as an internal control. Total RNA, 1 μg per lane, was separated on a 1% agarose/formaldehyde gel. After blotting onto a nylon membrane, the membrane was probed with digoxigenin-labeled anti-sense *GCRG123* cRNA. N, normal gastric tissue; T, tumor; 1, signet-ring cell carcinoma; 2, intestinal-type adenocarcinoma.

in the literature[15]. Multalin analysis revealed that *GCRG123* was located at nucleotides 2767-3215 of L1, at which the L1 ORF2 dwells (Figure 3). *GCRG123* was found to integrate in intron-17 and -23 of Rb (L11910), the 5' flanking region of IL-2 (X67285), and the 5' flanking region of clotting factor IX (X75349) genes (GenBank accession numbers in parentheses). BLAT analysis showed that *GCRG123* mapped to all the human chromosomes.

DISCUSSION

Our previous bioinformatic analysis of *GCRG123* showed that the predicted peptide sequence of *GCRG123* has 80% homology with a human lamin-like protein (GenBank accession number, AAA36178)[9], therefore, we predicted that *GCRG123* might be a gene that encoded a lamin-like protein. However, the results of the present study indicate that *GCRG123* tends to be an active LINE-1 family member.

Retrotransposable elements, such as L1, Alu and endogenous retroviruses, make up some 45% of human DNA[16]. L1 is one of the most successful retrotransposons and occupies 17% of DNA[17]. In the early stages, because of the lack of any obvious correlation with cellular functions, retrotransposable elements were considered to be mere remnants of our genetic evolution and given the name "junk DNA"[18]. However, recent data have revealed that some L1s do remain retrotransposition-competent. It has been shown that L1 is a functionally capable gene. For example, human embryonic stem cells can express endogenous L1 elements[19]. L1 can retrotranspose in neuronal precursors, and can consequently alter the expression of neuronal genes, which in turn, can influence neuronal cell fate *in vitro*[20]. L1 retrotransposition can also occur in non-dividing somatic cells[21].

A retrotransposition-competent, functional L1 element covers about 6.1 kb and contains a 5' untranslated region, a 1-kb ORF1 that encodes a protein (p40) with RNA-binding ability, followed by a 3.8-kb ORF2 that codes for a protein (p150) with endonuclease and reverse transcriptase (RT) activities, and a cysteine-histidine-rich domain. The 3' end of L1 is terminated by a short 3' untranslated region and a poly(A) tail[22-26]. L1 likely integrates into the genomic DNA by a process called target primed reverse transcription and shapes mammalian genomes through many different ways, such as retrotransposition, transduction and gene expression alteration[27]. Although the overwhelming majority of L1s (about 99.8%) are inactive because of 5' truncations, internal

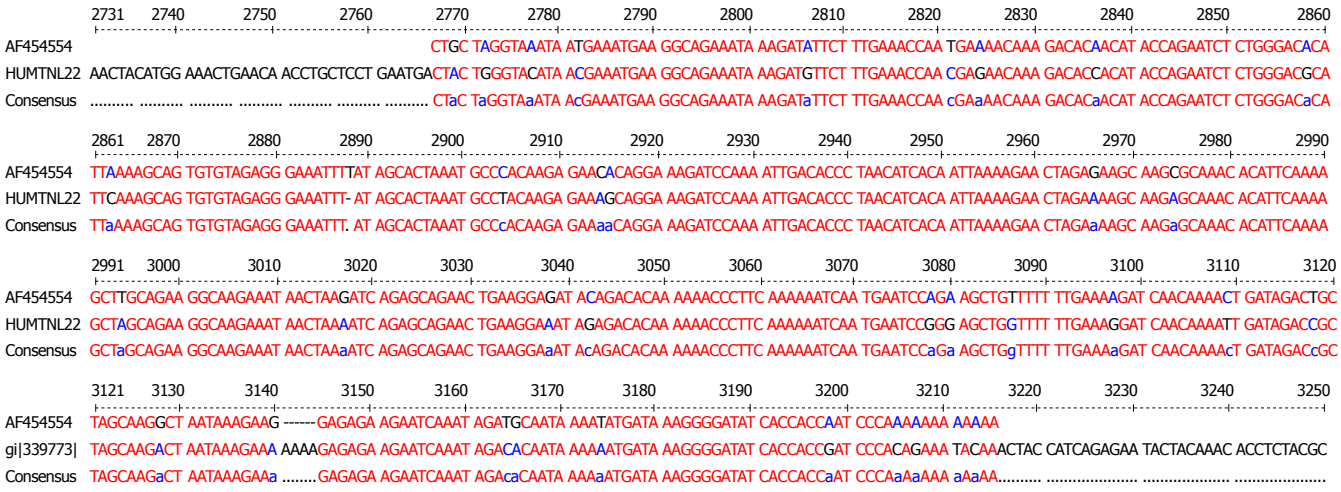


Figure 3 Multalin analysis of *GCRG123* (AF454554) and L1 (HUMTNL22). Red font represents high consensus, blue or black font represents low consensus.

rearrangements and mutations, 80-100 retrotransposition-competent L1s have been predicted in humans. Remarkably, 84% of the retrotransposition capability of these elements has been shown to be present in six highly active L1s[15].

It has been proposed that retrotransposons modulate the expression of specific genes through a transcriptional interference-based mechanism. To exert such effect on neighboring genes, retrotransposons need not necessarily be full-length, nor retrotransposition-competent, but simply endowed with functional promoter elements[28]. Previous studies have shown that some non-full length L1 elements (even shorter than 500 bp) may yield L1 products such as L1 RNA or active RT[29-31]. L1 RNAs are also involved in extensive RNA splicing that can radically alter the diversity of expressed RNA forms from these elements, as well as influence their impact on gene expression upon genomic insertion[32].

The state-of-the-art understanding of L1 described above prompted us to propose that *GCRG123* may be an active L1 family member, as supported by our findings. First, the *GCRG123* sequence shared 92% homology with the conserved sequence of the highly active L1s (hot L1s). Second, *GCRG123* can map to all chromosomes, which is similar to L1s. Third, *GCRG123* can integrate in the intron or 5' flanking region of some known genes, which suggests that it has the potential to adjust the function of these genes. Finally, *in situ* hybridization and Northern blot analysis at the mRNA level showed a distinct *GCRG123* expression pattern in different kinds of gastric mucosa cells.

A lot of evidence has indicated that L1 is involved in a wide spectrum of diseases, including cancer[28,33,34]. In colon cancer, the *APC* gene can be disrupted by somatic insertion of an L1 sequence into the last exon of the gene[35]. Takai *et al* have found that hypomethylation of L1 is detected in human hepatocellular carcinoma, but never in the surrounding liver tissues, whether or not liver cirrhosis is present[36]. Hypomethylation of L1 has also been revealed in urothelial carcinoma and prostate adenocarcinoma[37-39]. In malignant cells, there is a direct correlation between the hypomethylation of 5' L1 sequences and the presence of L1 proteins, which suggests that elements with hypomethylated 5' ends are transcriptionally active[40]. The hypomethylation of repetitive elements in cancer is directly linked to the neoplastic process and not a simple consequence of loss of growth control[41]. Previous studies have shown that L1 is essential for the proliferation of tumor cells. The functional knock-out of LINE-1 expression by RNAi reduces the proliferation and promotes the differentiation of tumorigenic human cells[29,42]. Up till now, no data about the relationship between L1 and stomach cancer have been reported. The present study on the distinct expression pattern of *GCRG123* in gastric signet-ring cell carcinoma may be the first to link an L1 family member to stomach oncogenesis.

During previous decades, several histological classification systems, such as those of Lauren, Nakamura and Ming, have been widely used for gastric adenocarcinoma. These systems agree that the intestinal/differentiated/expending type and diffuse/undifferentiated/infiltrative type differ greatly in terms of epidemiology, pathogenesis, genetic profile, and clinical outcome[8,43-46]. Signet-ring cell carcinoma belongs to the diffuse/undifferentiated/infiltrative type group. Nakamura has indicated that the morphological findings of undifferentiated type carcinoma under light and electron microscopy are similar to both the mucous cells of pyloric crypts and pyloric gland cells[44]. Interestingly, our study found that *GCRG123* was over-expressed in pyloric gland cells, epithelia (surface mucous cells) and signet-ring cells, which correlated with Nakagawa's description of undifferentiated carcinoma. The distinct *GCRG123* expression patterns in intestinal-type adenocarcinoma and signet-ring cell carcinoma also support the concept that these two types of carcinoma have different genetic profiles. *GCRG123* is over-expressed simultaneously in gastric epithelia, pyloric glands and signet-ring cell carcinoma, therefore, we believe that *GCRG123* might be closely associated with gastric mucous secretion. Further studies are required to confirm this speculation.

COMMENTS

Background

Evidence indicates that a retrotransposition-competent, functional L1 element is involved in a wide spectrum of diseases, including cancer. A previous study

has shown that a gene segment *GCRG123* is down-regulated in human gastric intestinal-type adenocarcinoma. Intestinal- and diffuse-type gastric cancers differ in their epidemiology, pathogenesis, genetic profile and clinical outcome.

Research frontiers

To modulate the expression of specific genes, retrotransposons, such as L1, need not necessarily be full-length, nor retrotransposition-competent, but simply endowed with functional promoter elements. Some non-full length L1 elements could yield L1 products such as L1 RNA or active RT. L1 is essential for the proliferation of tumor cells. The functional knock-out of L1 expression reduces cell proliferation and promotes cell differentiation.

Innovations and breakthroughs

GCRG123 may be an active L1 family member, because of its sequence homology with the conserved sequence of the highly active L1s and its integration in the intron or 5' flanking region of some known genes. *In situ* hybridization and Northern blot analysis showed that *GCRG123* was up-regulated in signet-ring cell carcinoma. Besides, *GCRG123* was also over-expressed in normal gastric epithelia and pyloric glands. The present study possibly represents the first to link an L1 family member to stomach oncogenesis.

Applications

Updated knowledge about L1 shifts the focus from protein-coding genes to repeated retroelements as mediators of abnormal biological processes. Functional L1 family members may be regarded as promising targets in the development of novel, differentiation-inducing approaches to cancer therapy. The distinct expression pattern of *GCRG123* in gastric signet-ring cell carcinoma may provide a new area to further understand the genetic characteristics of gastric signet-ring cell carcinoma.

Terminology

LINE-1: highly repeated sequences, 6-8 K base pairs in length, which contain RNA polymerase II promoters. They also have an ORF that is related to the RT of retroviruses, but they do not contain long terminal repeats. Copies of the LINE 1 (L1) family form about 17% of the human genome.

Peer review

This is an interesting article. The title reflects the major contents of the article. The abstract gives a clear delineation of the research background, objectives, materials and methods, results and conclusions. The methods are innovative and systematic. A detailed description has been provided. The results provide sufficient experimental evidence or data from which scientific conclusions can be drawn. The discussion is well organized and an overall theoretical analysis is given. The most current literature is cited. Overall, a it is well-written and well-researched article.

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RAPID COMMUNICATION

Synthesis of platelet-activating factor and its receptor expression in Kupffer cells in rat carbon tetrachloride-induced cirrhosis

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Abstract

AIM: To determine the platelet-activating factor (PAF) synthesis and its receptor expression in Kupffer cells in rat carbon tetrachloride-induced cirrhosis.

METHODS: Kupffer cells, isolated from the livers of control and CCl_4-induced cirrhotic rats, were placed in serum-free medium overnight. PAF saturation binding, ET-1 saturation and competition binding were assayed. ET-1 induced PAF synthesis, mRNA expression of PAF, preproendothelin-1, endothelin A (ETA) and endothelin B (ETB) receptors were also determined.

RESULTS: A two-fold increase of PAF synthesis (1.42 ± 0.14 *vs* 0.66 ± 0.04 pg/μg DNA) and a 1.48-fold increase of membrane-bound PAF (1.02 ± 0.06 *vs* 0.69 ± 0.07 pg/μg DNA) were observed in activated Kupffer cells of cirrhotic rats. The application of ET-1 to Kupffer cells induced PAF synthesis in a concentration-dependent manner in both cirrhotic and normal rats *via* ETB receptor, but PAF synthesis in the activated Kupffer cells was more effective than that in the normal Kupffer cells. In activated Kupffer cells, PAF receptor expression and PAF binding capacity were markedly enhanced. Activated Kupffer cells raised the [^{125}I]-ET-1 binding capacity, but changed neither the affinity of the receptors, nor the expression of ETA receptor.

CONCLUSION: Kupffer cells in the course of CCl_4-induced cirrhosis are the main source of increased PAF. ET-1 is involved endogenously in stimulating the PAF synthesis in activated Kupffer cells *via* ETB receptor by paracrine. ETA receptor did not appear in activated Kupffer cells, which may exacerbate the hepatic and extrahepatic complications of cirrhosis.

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Key words: Platelet activating factor; Kupffer cells; Receptor; Cirrhosis

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INTRODUCTION

Kupffer cells[1], resident macrophages in the liver, constitute the largest population of macrophages in the body and are critical effector cells for the defense against invading micro-organisms and foreign molecules. On the other hand, Kupffer cells in the pathogenesis of different types of liver injury[2-4] have been demonstrated to be involved in several models of liver damage induced by chemicals including carbon tetrachloride, alcohol and allyl alcohol[5,6]. The pathologic role of Kupffer cells has been confirmed by the attenuation of hepatotoxicity in terms of steatosis, inflammation, and necrosis in animals. It is hypothesized that Kupffer cells are activated in response to chemical administration and promote tissue damage by releasing biologically active mediators such as prostaglandins, TNFα, oxygen free radicals, and other proinflammatory mediators[7-9]. Platelet-activating factor (PAF) is the trivial name for a phospholipid called 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine that has unique phospholipid autacoid exhibiting diverse biological activities in various tissues and organs such as isolated lung, liver and brain, and different types of cells (platelet, neutrophils, endothelial cells, neural cells)[10-15]. These activities include platelet secretion and aggregation, broncho-constriction, increased vascular permeability, and systemic hypotension[16-18]. Previous studies demonstrate that PAF could raise portal vein pressure and stimulate glycogenolysis[19,20], the infusion of low doses of synthetic

PAF into the systemic circulation of normal rats caused hyperdynamic status, and PAF production may be dangerous in patients with portal hypertension[20,21]. PAF acts as both a multifunctional soluble proinflammatory agent and a specific membrance-bound adhesion molecule[22-24]. Since the biological effects of PAF are mediated by its specific receptor[25,26], almost all actions of PAF can be prevented by blocking its receptor with antagonists. PAF binding to its receptor induces release of arachidonic acid and produces cycloxygenase-derived eicosanoids[27], glycogenolysis, *etc.* which may mediate vasoconstriction and affect portal vein pressure and hemodynamics. Recent studies have shown that Kupffer cells possess a large number of high affinity PAF receptors which, when stimulated, synthesize and release several active lipid mediators such as eicosanoides and PAF[28]. Houssert *et al* showed that Kupffer cells contain mRNA for the endothelin B (ETB) receptor. However, they did not measure ET binding in Kupffer cells. PAF synthesis/release and its receptor expression in activated Kupffer cells are far from clear in carbon tetrachloride induced cirrhosis. The aim of this study was to elucidate the characteristics of PAF synthesis/release and its receptor expression of the Kupffer cells in rat carbon tetrachloride-induced cirrhosis.

MATERIALS AND METHODS

Establishment of animal model of liver cirrhosis

The experimental protocols were approved by the Animal Care and Use Committee of the Institute of Infectious Diseases of PLA. Cirrhosis was induced in male Sprague-Dawley rats (230-250 g) by intraperitoneal injection of CCl_4 (0.15 mL/kg twice a week for 8 wk). Control rats received injection of the carrier (peanut oil). Rats were allowed access to water containing phenobarbital (0.4 g/L) as the only drink source throughout the induction four days before the CCl_4 treatment. This treatment caused complete distortion of the hepatic architecture due to excessive deposition of the extracellular materia (ECM), portal hypertension, hyperdynamic state and ascites in more than 70% of rats.

Preparation of Kupffer cells

Kupffer cells were prepared from carbon tetrachloride induced male Sprague-Dawley rats . Briefly, after the collagenase (type Ⅳ from clostridium histoticum, 0.025%)/protease (type XIV, from streptomyces griseus, 0.05%) digestion of liver and removal of the hepatocytes and cells debri by low speed centrifugation, Kupffer cells and endothelial cells were purified from other nonparenchymal cells by density gradient centrifugation using 30%(W/V) metrizamide. Kupffer cells were then isolated with a modified centrifugal elutriation procedure. The viability of the Kupffer cells was greater than 95% as determined by trypan blue exclusion. Finally, Kupffer cells were suspended in Williams' medium E (Sigma) containing 10% heat-inactivated fetal calf serum, 5000 U/mL penicillin and 5000 μg/mL streptomycin and plated onto 12- well culture plates at a density of 0.5×10^6 cells/cm^2, and incubated in an atmosphere of 95% air/5% CO_2 at 37℃. The medium was renewed after incubated for 3 h and the cells were used for experiments after an overnight incubation. Purity of the cells determined was greater than 95% by immunostaining with ED2 (Kupffer cells), desmin (stellate cells) and factor Ⅷ related antigen (endothelial cells).

Determination of PAF level in associated Kupffer cells and medium

Lipid extraction: Lipid in associated Kupffer cells and medium was extracted according to the method of Bligh & Dyer. In brief, 1 mL medium was added with methanol (2.5 mL) and chloroform (1.25 mL) was prepared (methanol: chloroform:medium is 2:1:0.8 v:v:v). The mixture was kept for 1 h at room temperature, and added with chloroform (1.25 mL) and water (1 mL), (methanol: chloroform:water is 1:1:0.9 v:v:v). The new mixture was kept for 1 h at room temperature and centrifuged at 1200 × *g* for 15 min. The chloroform layer was dried under a stream of nitrogen at 35℃. In associated Kupffer cells, methanol (2.5 mL), chloroform (1.25 mL) and water (1 mL) (methanol:chloroform:water is 2:1:0.8 v:v:v) were added into the medium.

Purification of PAF: Amprep silica mini-column was used according to the instructions of its manufacturer. In brief, residue was dissolved in 200 μL chloroform and applied to a Bond Elut SI column. The column was washed with 3 mL chloroform, 2 mL chloroform-methanol (3:2, v:v) and 3 mL chloroform-methanol-28% aqueous ammonia (70:85:7, v:v:v), and eluted with 2 mL chloroform-methanol-28% aqueous ammonia (50:50:7, v:v:v). Eluate was evaporated till dried, and the residue was dissolved in 200 μL saline containing 0.1% Triton X-100.

Determination of PAF: Platelet activating factor [^{3}H] scintillation proximity assay (SPA) system kit (Amersham Pharmacis Biotech, USA) was used according to the instructions of the manufacturer.

Morphometric analysis: The activated Kupffer cells were incubated for a day and stained by the method of immunohistochemistry to recognize membrane-bound PAF within activated Kupffer cells. The primary antibody used was a sheep anti-PAF polyclonal preparation [platelet activating factor [^{3}H] scintillation proximity assay (SPA) system kit, Amersham Pharmacis Biotech, USA] The primary antibody was incubated overnight and followed by 0.5 h with secondary antibody in streptavidin-horseradish peroxidase at 1:2500 dilution (DAKO LSAB+ kit, peroxidase, KO679).

Determination of mRNA expression of PAF receptor in activated Kupffer cells

The levels of the low abundance mRNA transcripts of PAF receptor were determined by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) using the following procedure. β-actin mRNA expression was determined using the same amount of cDNA as β-actin mRNA. RNA was isolated from the livers using an

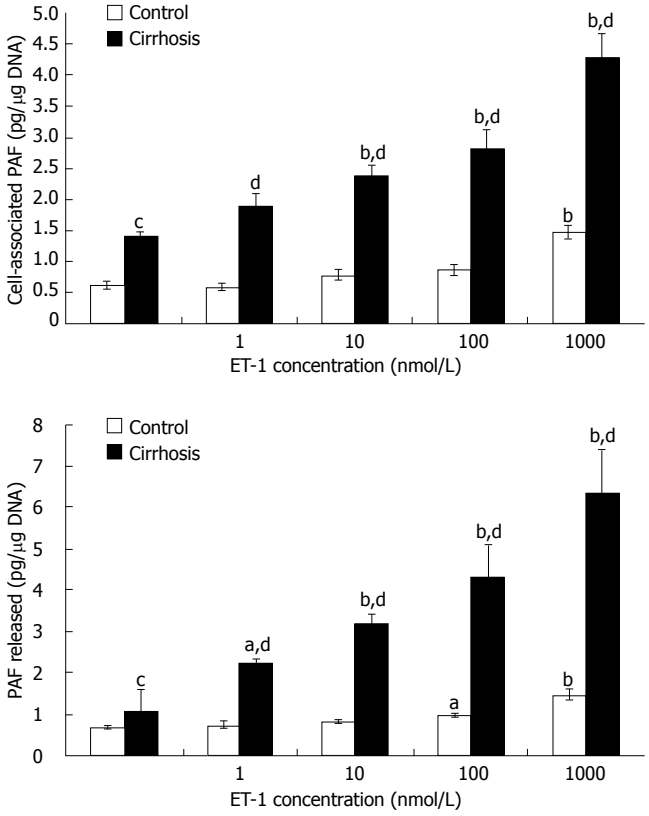


Figure 1 Effect of ET-1 on PAF synthesis in cultured activated Kupffer cells. Kupffer cells from control and cirrhotic rats were cultured and stimulated with increasing concentrations of ET-1 for 15 min in serum-free medium. Cell-associated and released PAF levels were determined as explained in the Methods section. Each point represents an average of triplicate values ± SEM from a representative of two experiments. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* vehicle; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* control.

RNA isolation kit (ToTALLY RNA™, Ambion, Austin, TX) as per the instructions of the manufacturer. The concentration of RNA was determined by measuring A260, and the purity was checked by the A260/A280 ratio (greater than 1.8). Two μg total RNA obtained from Kupffer cells was used for the preparation of cDNA by reverse transcriptase-PCR. The PCR primers for PAF cDNA and β-actin cDNA are: 5'-GCCACAACACAGAG GCTTGA-3' (F) and 5'-TCCATTGCTCTGGGCAGGAA -3' (R) (121 bp); 5'-TTCTACAATGAGCTGCGTGTG-3' (F) and 5'-TTCATGGATGCCACAGGATTC-3' (R) (561 bp), respectively. DNA equivalent of 5 ng original RNA was used in PCR. The reaction mixture (50 μL) contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 20 pmol PCR primer and 2 units of Platinum Taq DNA polymerase (GIBCO-Invitrogen, Carlsbad, CA). The reaction for PAF receptor was run for 35 cycles as follows: denaturation at 94℃ for 1 min, annealing at 60℃ for 30 s, and extension at 72℃ for 30 s. The PCR products were resolved in a 2.5% agarose gel and stained with 1 × SYBR Green I (FMC Biproduct, Rockland, ME). The gels were scanned under blue fluorescence light using a phosphorimager and the band intensity was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Determination of PAF receptor in activated Kupffer cells

After an overnight incubation, Kupffer cells were washed and incubated in 50 mmol/L Tri-HCl , pH 7.2 (5 mmol/L $MgCl_2$, 125 mmol/L choline chloride, 0.25% BSA) containing 0.125-32 nmol/L ^{3}H -PAF (151 Ci/mmol, UK) ± 10 μmol/L unlabelled PAF (C16) (saturation binding) at 22℃ for 3 h. The reaction was terminated with the addition of 5 mL ice-cold assay buffer, the cells were washed in assay buffer and digested with 5% SDS at 60℃ for 30 min. Radioactivity was detected with 5 mL β-scintillation solution. The results were expressed as CPM of ^{3}H -PAF bound per μg protein.

Determination of mRNA expression of preproET-1 and ET-1 receptors in Kupffer cells

Semiquantitative RT-PCR assay was employed to assess the relative levels of preproET-1, ET-1 receptors in Kupffer cells. β-actin mRNA expression was determined using the same amount of cDNA. The PCR primers specific for prproET-1 cDNA are: 5'-CCAACTCTGGGCTCTCCA TGCTGG-3' (F) and 5'-GAATGGCACTGTGTCTCTG CTCTC-3' (R) [241 bp]; for ETA cDNA: 5'-CCCTTCGA ATACAAGGGCGA-3' (F) and 5'-GAAGAGGGAACC AGCACAGTTTCTACTTCTGC-3' (R) [291 bp]; and for ETB cDNA: 5'-GGCTGTTCAGTTTTCTACTTCTGC-3' (F) and 5'-AGAATCCTGCTGAGGTGAAGG-3' (R) [210 bp]. PCR amplification reaction was performed as follows. PCR products were resolved in a 1.2% agarose gel and stained with 1 × SYBR Green I (FMC Biproduct, Rockland, ME, USA). The other procedures were the same for the PAF receptor mentioned above.

Determination of ET-1 receptor in Kupffer cells

After an overnight incubation, activated Kupffer cells were washed with HBSS containing 10 mmol/L HEPES, pH 7.4, and 0.1% bovine serum albumin (HBSS/BSA) and then placed in the medium containing 6.25-800 pmol/L [^{125}I]-ET-1 ± 1 μmol/L unlabelled ET-1 (saturation binding). In the competition binding assay, cells were incubated with 50 pmol/L [^{125}I]-ET-1 ± 1 μmol/L unlabelled ET-1, 5 μmol/L ETA antagonist BQ-123 or 5 μmol/L ET B antagonist BQ-788. After incubation at 22℃ for 3 h, the cells were washed with HBSS/BSA and digested with 0.75N NaOH for determination of radioactivity. Specific binding of [^{125}I]-ET-1 was different in cell-associated radioactivity in the presence and absence of 1 μmol/L unlabelled ET-1.

Statistical analysis

The values are presented as averages of triplicate determinations ± SEM. Each experiment was repeated at least three times using cells from different animals. Student *t* test was employed for statistical comparison of the paired samples. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Effect of ET-1 on PAF synthesis in activated Kupffer cells

Basal release of PAF was increased by about two-fold in cirrhotic rat Kupffer cells as compared with the control

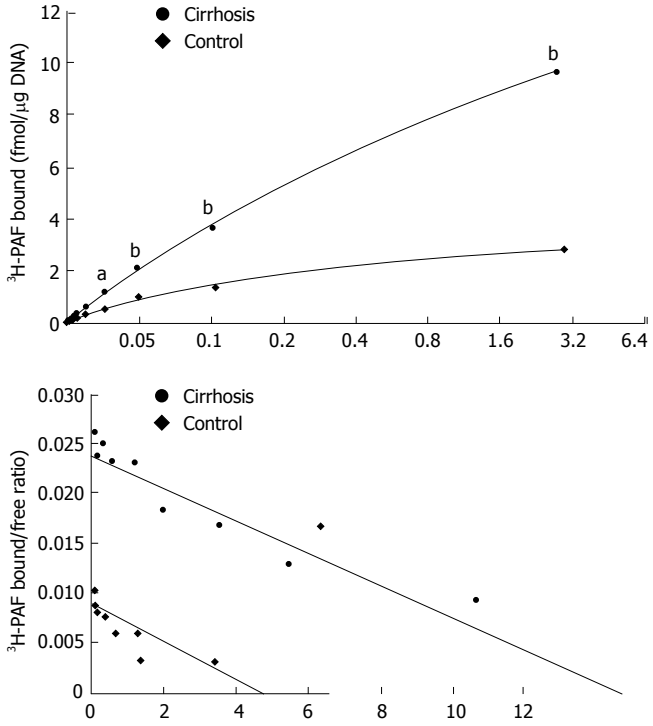


Figure 2 **A**: Saturation curve binding to cirrhotic and normal rat Kupffer cells. Cells were used after overnight culture for receptor binding assay as described in the Method section. ^{3}H-PAF at a concentration between 0.125 and 3.2 nmol/L was incubated with Kupffer cells in presence or absence of 5 μmol/L unlabeled PAF at 22℃ for 3 h. $^aP < 0.05$, $^bP < 0.01$ *vs* control; **B**: Scattered blot analysis of ^{3}H-PAF binding to cirrhotic and normal rat Kupffer cells. The results were analyzed by scatchard plot. Cirrhosis: R = 0.99, Kd = 2.08 nmol/L, Bax = 27.1882 ± 2.0885 fmol/mg. DNA; Control: R = 0.96, Kd = 1.57 nmol/L, Bax = 4.4024 ± 0.3155 fmol/mg. DNA.

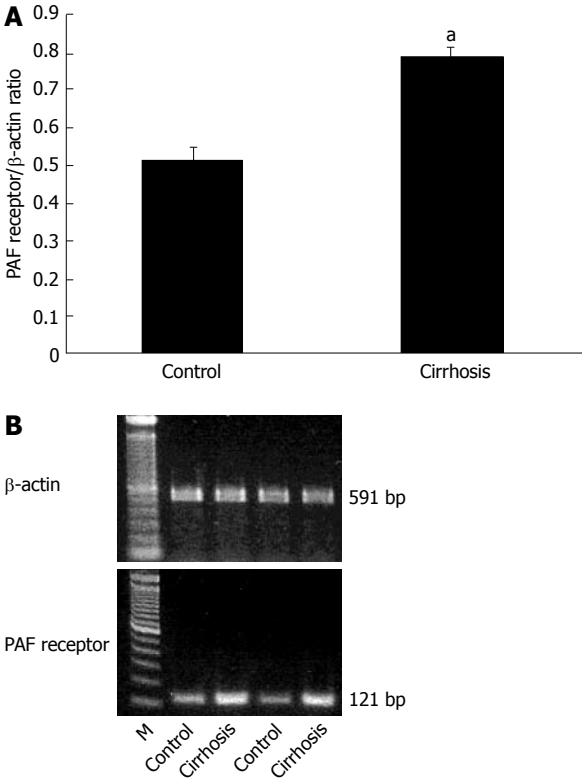


Figure 3 **A**: The mRNA expression of cirrhotic rat Kupffer cell PAF receptor. Expression of β-actin mRNA (β-actin control: 90 311.14 ± 3882.67; cirrhosis: 110 958.2 ± 1484.58). $^aP < 0.05$ *vs* control; **B**: Character of the PAF receptor expression in cirrhotic Kupffer cells. Total RNA was extracted from the cells for determination of PAF receptor mRNA expression by semiquantitative reverse transcriptase polymerase chain reaction assays as described in the Method section.

(1.42 ± 0.14 *vs* 0.66 ± 0.04 pg/μg DNA, $P < 0.01$), while basal release of membrane-bound platelet-activating factor was only increased by about 1.48-fold (1.02 ± 0.06 *vs* 0.69 ± 0.07 pg/μg DNA, $P < 0.01$) (Figure 1). Interestingly, activated Kupffer cells appeared to release more PAF than membrane-bound platelet-activating factors. Application of ET-1 to rat Kupffer cells induced the PAF synthesis in both control and activated Kupffer cells (Figure 1). However, the effect was more pronounced in the latter. Significantly induced PAF synthesis occurred at 100 nmol/L ET-1 in control and cirrhotic Kupffer cells, and therefore, statistical significance was observed at 1-1000 nmol/L ET-1. Immunochemical detection of ET-1 induced PAF synthesis in cirrhotic and normal Kupffer cells showed that cytoplasm PAF was stained, strongly and increased in a concentration-dependent manner (B, D pulsed ET-1 100 nmol/L), and the cytoplasm of PAF staining in activated Kuppffer cells increased more significantly than that in the controls.

PAF receptor expression in cirrhotic Kupffer cells

Scattered blot analysis of the saturation binding data revealed about a six-fold increase in ^{3}H -PAF binding capacity in cirrhotic Kupffer cells as compared with the controls (Bmax of 27.19 ± 2.09 *vs* 4.40 ± 0.32 fmol/μg. DNA). The affinity of the receptors was not altered in the cirrhosis (Kd of 2.08 ± 0.22 nmol/L in the cirrhosis and 1.57 ± 0.17 nmol/L in the control) (Figure 2A and B). In cirrhosis, PAF receptor expression of activated Kupffer cells increased 1.5-fold as agaist the control (0.78 ± 0.03 *vs* 0.52 ± 0.03) (Figure 3A and B), which was consistent with PAF receptor saturation binding data.

Preproendothelin-1 and ET-1 receptor expression in cirrhotic Kupffer cells

Figure 4 illustrates the scattered blot of the results of [^{125}I]-ET-1 saturation binding assays in cirrhotic Kupffer cells. Activated Kupffer cells increased their binding capacity by about two-fold as compared with the control (Bmax of 1639 ± 27.71 *vs* 820.4 ± 28.75 fmol/μg DNA). No change was observed in the affinity of the receptors in the cirrhosis (Kd of 37.72 ± 6.08 pmol/L in cirrhosis and 22.79 ± 3.46 pmol/L in the control). As shown in Figure 5, in 50 pmol/L [^{125}I]-ET-1, the [^{125}I]-ET-1 specific binding capacity increased by about 1.7-fold (Bmax of 0.34 ± 0.02 *vs* 0.20 ± 0.01 fmol/μg DNA). In the cirrhotic Kuppfer cells, the number of ET-1 receptor was larger than that of the control. ETA receptor antagonist BQ-123 did not affect [^{125}I]-ET-1 specific binding (specific binding: 0.34 ± 0.02 fmol/μg DNA; BQ-123: 0.25 ± 0.02 fmol/μg DNA in cirrhosis; specific binding: 0.22 ± 0.01 fmol/μg DNA, BQ-123: 0.18 ± 0.01 fmol/μg DNA in control), but ETB receptor antagonist BQ-788 significantly blocked [^{125}I]-ET-1 specific binding (cirrhosis: 0.08 ± 0.003 fmol/μg DNA; control: 0.09 ± 0.002 fmol/μg DNA). This revealed that activated Kupffer cells only increased the number of ETB

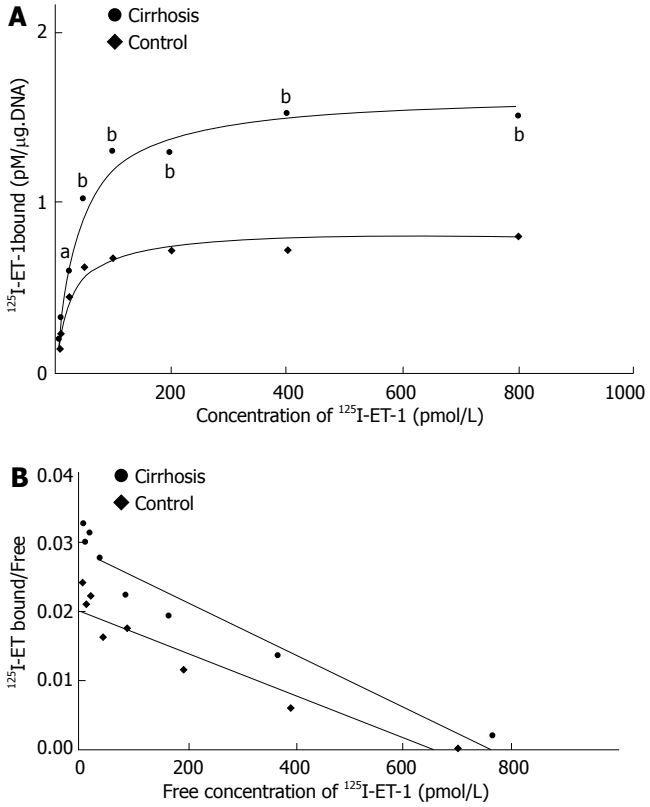


Figure 4 **A**: Saturation curve of ^{125}I-ET-1 binding to cirrhotic and normal rat Kupffer cells. ^{125}I-ET-1 at a concentration between 6.25-800 pmol/L was incubated with Kupffer cells (1×10^6), in presence or absence of 2 μmol/L unlabeled ET-1 at 20℃ for 3 h; **B**: Scattered blot analysis of ^{125}I-ET-1 binding to cirrhotic and normal rat Kupffer cells. The result was analyzed by scatchard plot. Cirrhosis: R = 0.98, Kd = 37.72 pmol/L, Bax = 1639 ± 67.7 fmol/mg. DNA; Control: R = 0.94, Kd = 22.79 pmol/L, Bax = 820.4 ± 28.7 fmol/mg. DNA.

receptor, but never changed the ET-1 receptor expression. Figure 6 shows the characteristics of the expression of preproendothelin-1 and ET-1 receptor subtypes in the Kupffer cells of cirrhotic rats. The activated Kupffer cells neither expressed preproendothelin-1 nor changed the expression of ET-1 receptor subtypes, they only expressed ETB receptor which was increased by 166.07% as compared with the control (0.34 ± 0.01 *vs* 0.21 ± 0.01).

DISCUSSION

Elucidation of the interactions between activated Kupffer cells and ET-1 as a method for detecting hepatic cirrhosis and attendant hemodynamic abnormalities in pathophysiology, has been a topic of intensive investigations in the recent years. Previous studies have demonstrated that ET activates both phosphoinositide metabolism and prostaglandin synthesis in rat Kupffer cells[30] and ET-1 stimulates PAF synthesis in Kupffer cells[29], while the current study has demonstrated that ET results in biosynthesis and release of PAF[31] in cultured rat Kupffer cells. The diversity of ET actions in Kupffer cells might be explained by the existence of more than one receptor subtype or activation of more than one signal transduction pathway after endothelin binding. Houssert[32] found that Kupffer cells contain mRNA for the ETB receptor, however they did not measure ET binding in Kupffer cells. Based on the growing body of evidence regarding hepatic cirrhosis, it is evident that Kupffer cells act as

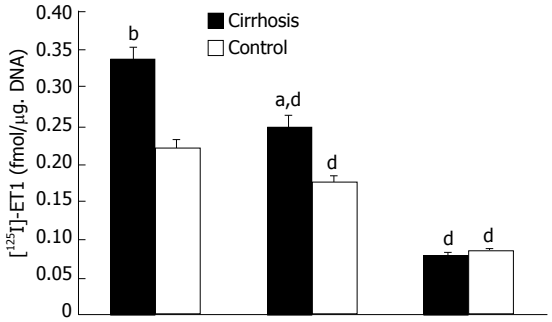


Figure 5 Competition binding assay (50 pmol/L ^{125}I-ET-1 ± 1 μmol/L unlabelled ET-1, 5 μmol/L ETA antagonist BQ-123 or 5 μmol/L ETB antagonist BQ-788). Specific binding of [^{125}I]-ET-1 was different in cell-associated radioactivity in the presence and absence of 1 μmol/L unlabelled ET-1. $^aP < 0.05$, $^bP < 0.01$ *vs* control; $^dP < 0.01$ *vs* [^{125}I]-ET-1.

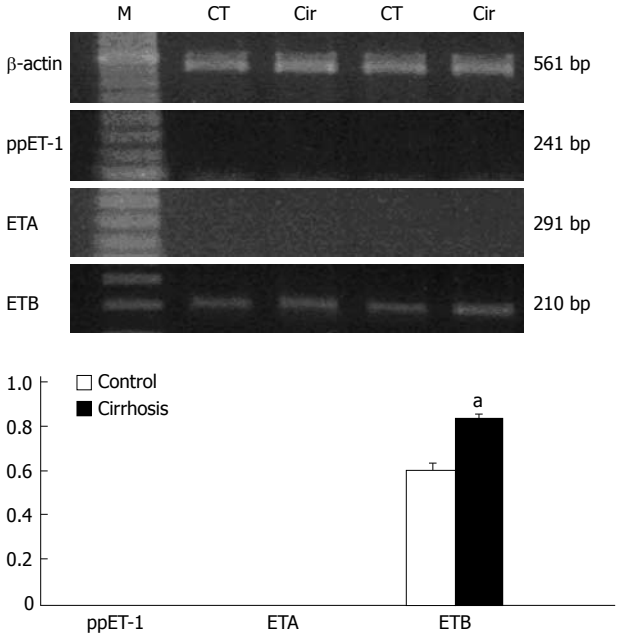


Figure 6 Characteristics of the preproendothelin-1 and ET-1 receptor subtype expression in the cirrhotic rat Kupffer cells. RNA was extracted from the cells for determination of preproET-1 and ET-1 receptor subtypes mRNA expression by semi-quantitative reverse transcriptase polymerase chain reaction assay. Graphical presentation of data as standardized relative to expression of β-actin mRNA (β-actin control: 90 311.14 ± 3882.67; cirrhosis: 11 095.82 ± 1484.58). $^aP < 0.05$ *vs* control.

critical effector cells in the pathogenesis of cirrhosis and hemodynamic abnormalities[33]. Our study has demonstrated that PAF could lower the systemic arterial pressure and raise the portal pressure[34,35], so PAF is a principal mediator of hyperdynamic circulation and portal hypertension. As illustrated in Figure 1, activated Kupffer cells enhanced the synthesis of PAF, and appeared to secrete more PAF than membrane-bound platelet-activating factor. PAF synthesis was increased in a concentration-dependent manner in both cirrhotic and normal rat Kupffer cells. In the present study, enhanced synthesis of PAF by activated Kupffer cells may account for the increased hepatic concentration and PAF level. The increase (Figures 2 and 3) in activated Kupffer cells demonstrated that PAF had an autocrine function on activated Kupffer cells. PAF increase in activated Kupffer

cells has been implicated to be an important mediator of hyperdynamic circulation associated with liver cirrhosis. In agreement with the previous reports in cirrhotic patients, increased blood PAF levels were observed in CCl_4-induced cirrhotic rats[36]. The results of the present study suggest that the enhanced synthesis and release of PAF by activated Kupffer cells may contribute to its elevated circulating concentration in cirrhotic rats. The basal levels of cell-associated and secreted PAF were significantly higher in activated Kupffer cells than in the controls. Because the biological effects of PAF are mediated by its specific receptor, almost all actions of PAF can be prevented by blocking its receptor antagonists. The binding of PAF to its receptor may induce the release of arachidonic acid and PAF, and the vasoconstriction, portal vein pressure and hyperdynamic circulation may be mediated and influenced by eicosanoids.

ET-1 induced PAF synthesis in activated Kupffer cells increased in a concentration-dependent manner, revealing that endogenously produced ET-1 can increase the hepatic concentration and PAF levels in cirrhosis. The activated Kupffer cells did not express preproendothelin-1, either, indicating that ET-1 stimulates PAF synthesis in activated Kupffer cells by paracrine. Clearly, Kupffer cells are major contributors to the increased hepatic concentration and PAF level in cirrhosis.

It has been known that Kupffer cells contain only ETB receptor. The ET-1 saturation and competition binding assay show that ET-1-induced increase in PAF synthesis was mediated *via* ETB receptor. Activated Kupffer cells enhanced the binding capacity, but did not change the affinity of the receptors (Figures 4 and 5), although the number of PAF receptors was increased. Activated Kupffer cells did not express ETA receptor (Figure 6). As a result of hepatic injury, ETA receptor did not appear in activated Kupffer cells. In cirrhosis, activated Kupffer cells did not change the character of ET-1 receptor expression.

In conclusion, activated Kupffer cells in the course of CCl4-induced cirrhosis are the main source of increased PAF; endogenously, ET-1 can stimulate PAF synthesis in activated Kupffer cells via ETB receptor by paracrine; and as a result of hepatic injury, ETA receptor did not appear in activated Kupffer cells. The PAF receptor expression in activated Kupffer cells and PAF binding capacity are markedly increased in cirrhosis, which may exacerbate the hepatic and extrahepatic complications of cirrhosis.

COMMENTS

Background

Kupffer cells in the pathogenesis of different types of liver injury have been demonstrated to be involved in several models of liver damage induced by chemicals including carbon tetrachloride, alcohol and allyl alcohol. The high affinity platelet-activating factor (PAF) receptors, when stimulated, synthesize and release several active lipid mediators including eicosanoides and PAF. Kupffer cells have been shown to contain mRNA for the ETB receptor. However, no ET binding was measured in Kupffer cells. PAF synthesis/release and its receptor expression in activated Kupffer cells is far from clear in the carbon tetrachloride induced cirrhosis. Kupffer cells are activated in response to chemical administration and promote tissue damage by releasing biologically active mediators such as prostaglandins, TNFα, oxygen free radicals, and other proinflammatory mediators. PAF raised portal vein pressure and stimulated glycogenolysis, and PAF production may be dangerous in patients with portal hypertension as reported previously. The biological effects of PAF are mediated by its specific receptor, therefore, almost all actions of PAF can be prevented by blocking its receptor with antagonists. PAF binding its receptor induces release of arachidonic acid and production of cycloxygenase-derived eicosanoids, glycogenolysis, etc. which may mediate vasoconstriction and affect portal vein pressure and hemodynamics.

Research frontiers

Elucidation of the interactions between activated Kupffer cells and ET-1 as the mechanism detecting hepatic cirrhosis and attendant hemodynamic abnormalities in pathophysiology, has been a topic of intensive investigations in the recent years.

Innovations and breakthroughs

This paper has confirmed that expression of PAF receptor in activated Kupffer cells and PAF binding capacity are markedly increased in cirrhosis, which may exacerbate the hepatic and extrahepatic complications of cirrhosis.

Applications

Based on the result of this research, we can decrease the synthesis of PLT in Kupffer cells by suppressing the secretion of ET-1, or blocking its receptor, which may lighten or defer the hepatic and extrahepatic complications of cirrhosis.

Terminology

Kuppfer cells, resident macrophages in the liver, constitute the largest population of macrophages in the body and are critical effector cells for the defense against invading micro-organisms and foreign molecules. Platelet-activating factor (PAF) is the trivial name for a phospholipid called 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine that has unique phospholipid autacoid exhibiting diverse biological activities on various tissues and organs such as isolated lung, liver and brain, and different types of cells (platelet, neutrophils, endothelial cells, neural cells). ET-1 stimulates PAF synthesis in Kuffer cells.

Peer review

The data presented by the authors strongly supports the hypothesis that Kupffer cells in cirrhotic livers are a main source of PAF, which contribute significantly to secondary signs of cirrhotic liver like portal hypertension. The topic is very interesting, and the experiments performed mainly prove the conclusions of the authors.

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RAPID COMMUNICATION

Relationship and significance between anti-β2-glycoprotein Ⅰ antibodies and platelet activation state in patients with ulcerative colitis

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Received: September 26, 2007 Revised: December 5, 2007

Abstract

AIM: To study the relationship between anti-β2-glycoprotein Ⅰ (aβ2GP Ⅰ) antibodies and platelet activation state in patients with ulcerative colitis (UC) and its significance.

METHODS: Peripheral blood samples were collected from 56 UC patients (34 males and 22 females, aged 43.5 years, range 21-66 years), including 36 at active stage and 20 at remission stage, and 25 sex-and age-matched controls. The level of aβ2GP Ⅰ was measured by ELISA. The platelet activation markers, platelet activation complex-Ⅰ (PAC-Ⅰ) and P-selectin (CD62P) were detected by flow cytometry.

RESULTS: The A value for IgG aβ2GP Ⅰ in the active UC group was 0.61 ± 0.13, significantly higher than that in the remittent UC and control groups (0.50 ± 0.13 and 0.22 ± 0.14, $P < 0.01$). There was a significant difference between the two groups ($P < 0.01$). The A value for IgM aβ2GP Ⅰ in the active and remittent UC groups was 0.43 ± 0.13 and 0.38 ± 0.12, significantly higher than that in the control group (0.20 ± 0.12, $P < 0.01$). However, there was no significant difference between the two groups ($P > 0.05$). The PAC-Ⅰ positive rate for the active and remittent UC groups was 30.6% ± 7.6% and 19.6% ± 7.8% respectively, significantly higher than that for the control group (6.3% ± 1.7%, $P < 0.01$). There was a significant difference between the two groups ($P < 0.01$). The CD62P positive rate for the active and remittent UC groups was 45.0% ± 8.8% and 31.9% ± 7.8% respectively, significantly higher than that for the control group (9.2% ± 2.7%, $P < 0.01$). There was a significant difference between the two groups ($P < 0.01$). In the active UC group, the more severe the state of illness was, the higher the A value for IgG aβ2GP Ⅰ was, and the positive rate for PAC-I and CD62P was positively correlated with the state of illness (Faβ2GP Ⅰ = 3.679, $P < 0.05$; FPAC-I (%) = 5.346, $P < 0.01$; and FCD62P (%) = 5. 418, $P < 0.01$). Meanwhile, in the same state of illness, the A value for IgG aβ2GP Ⅰ was positively correlated to the positive rates for PAC-I and CD62P.

CONCLUSION: aβ2GP Ⅰ level, platelet activation state and their relationship of them are closely correlated with the pathogenesis and development of UC.

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Key words: β2-glycoprotein Ⅰ; Anti-β2-glycoprotein Ⅰ antibodies; Ulcerative colitis; Platelet activation; Hypercoagulation

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INTRODUCTION

β2-glycoprotein Ⅰ (β2GP Ⅰ) contains 150-200 mg/L glycoprotein in plasma[1] and is synthesized by hepatocytes[2]. It participates in fat transfer and metabolism due to its lipotropy[3], and is also known as apolipoprotein H (apoH)[4]. It was reported that β2GP Ⅰ is an immunogen in antiphospholipid syndrome (APS)[5-10]. Antiphospholipid antibody is the only autoantibody that induces blood into hypercoagulability state, just like one bridge between two physiology systems, immunity and coagulation, which may cause formulation of arterial and venous thrombosis with

unknown reasons[11-15]. β2GP I is known as a target antigen of antiphospholipid antibody[16-19]. Although ulcerative colitis (UC) is an inflammatory bowel disease (IBD) with unknown reasons, it is closely related to immunity, thrombosis formulation and blood hypercoagulability state[20-24]. To research the possible mechanism of blood hypercoagulability state, we measured the aβ2GP I level and detected the platelet activation state in serum of UC patients.

MATERIALS AND METHODS

Materials

Heparin sepharose CL-6B and sephadex G-100 were from Swedish Pharmacia Company. Flow cytometer (FACSort Calibur Type) was from American Becton Dickion Company and MB-Ⅲ enzyme mark instrument was from Beijing New Technology Institute. Fluorescein isothiocyanate labeled against platelet activation GPI1b/Ⅲa (PAC-I FITC) fibrinogen receptor McAb, phycoerythrin labeled anti-P-selectin (CD62P PE) McAb, peridinin chlorophyll protein labeled platelet glycoprotein Ⅲ (CD61 per-cp) McAb, negative control RGDS as a PAC-I blocker, phycoerythrin labeled mouse IgG (Mouseγ1PE), FACS flow TM sheath fluid were all from American Becton Dickion Company. Horseradish peroxidase labeled goat anti-human IgG was a product from Canadian Anogen, and horseradish peroxidase labeled goat anti-human IgM was from Huamei Company. The rest agents used were of national analytical purity.

Patients

Fifty-six patients attending to No. 1 and 2 Hospitals Affiliated to Jilin University from September 2003 to December 2004 were included in the study as UC group. Of the 56 UC patients (34 males and 22 females at the age of 21-66 years with a mean age of 43.5 years), 36 had active UC, 20 had remittent UC. The average disease course was 18 mo. All the cases were diagnosed based on the diagnostic criteria for inflammatory bowel disease (IBD) revised at the National Inflammatory Bowel Disease Conference held in 2000. Of which, 47 patients were diagnosed based on the findings in clinical, endoscopic and pathologic examinations, 9 patients were diagnosed based on the findings in clinical and endoscopic examinations. None of the patients had hypercoagulability state in the heart, liver, kidney, pulmonary and cerebrovascular diseases. The patients did not take any drugs affecting coagulation and fibrinolytic systems 4 wk before their diagnosis. Twenty-five healthy persons (15 males and 10 females at the age of 22-58 years with a mean age of 42.5 years) served as the control group. There was no significant difference in sex and age between the two groups.

Measurement of β_2GP I level

Five microlitre of 700 mL/L perchloric acid was added into 200 mL human plasma, stirred for 15 min at 4℃, centrifuged at 12000 r/min for 20 min at 4℃, dialyzed (0.02 mol/L Tris-HCl, pH 8.0, 0.03 mol/L NaCl) and concentrated by heparin sepharose CL-6B affinity chromatography. Linear gradient elution (0.03-0.35 mol/L NaCl) was performed to collect about 0.25 mol/L elution, which was dialyzed (0.02 mol/L Tris-HCl, pH 8.0, 0.03 mol/L NaCl) and concentrated by heparin sepharose CL-6B affinity chromatography. Linear gradient elution was performed (0.15-0.35 mol/L NaCl), and the elution peak containing β2GP I was collected and concentrated. The sample was dialyzed (0.02 mol/L Tris-HCl, pH8.0, 0.03 mol/L NaCl) and concentrated. Sephadex G-100 was separated, elution peak containing β2GP I was collected, dialyzed (0.02 mol/L Tris-HCl, pH 8.0, 0.03 mol/L NaCl) and concentrated. Protein was determined with the Lowry method and protein purity was assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and ultraviolet scanning.

Detection of aβ_2GP I by ELISA

IgG aβ2GP I and IgM aβ2GP I were detected in each serum sample. Multi-wells were set for the two antibodies (n = 3). Enzyme labeled 96-well plates were disposed with ^{60}Co. Their surface was oxygenized and 100 μL coating solution containing 4 μg/mL β2GP I was added and stored at 4℃ overnight. The coating solution was recycled, the plates were washed three times(3 min each time) with phosphate-buffered solution-Tween 20 (PBS-T) and 6 g/L gelatin-PBS-T was added (100 μL per well) and sealed at 37℃ for 1 h. The plates were washed three times (3 min each time). The diluted serum sample was added (100 μL per well) and incubated at 37℃ for 2 h. The plates were washed three times (3 min each time) with PBS-T. The two antibodies were discarded, the plates were washed three times (3 min each time) with PBS-T. Stained liquid o-phenylene diamine was added (100 μL per well) and reacted with shade for 5 min, 2 mol/L H_2SO_4 was added (50 μL per well) and shaken evenly. The reaction was terminated and optical density value at 492 nm was detected with enzyme mark instruments.

Detection of platelet activation state in UC patients by flow cytometry

Three mL venous blood was taken. The first 2 mL mixed with tissue fluid was discarded, and the remained 1 mL was added into anticoagulative tubes, shaken and mixed evenly. PAC-I FITC, CD62P PE, CD61 per-cp (20 μL each) were added into experimental tubes for 10min. PAC-I FITC, IgG1PE, CD61 per-cp (20 μL each) and 10 μL RGDS were added into the control tubes, and 5 μL mixed blood was shaken evenly and added into the tubes separately. After incubated for 20 min at the room temperature, PBS (pH 7.2) was added, shaken evenly for 30 min and stored at a cool dark place.

Statistical analysis

SPSS 11.5 statistical software was used to establish database and analyze the data. Comparison between groups was made by t test and comparison between multi-independent samples was carried out by One-way ANOVA, related analysis was performed by Pearson analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Extraction and purity of β_2GP I

β2GP I was detected as one band by three-step

Table 1 Detection results of aβ2GP I in patients among groups (A_{492}, mean ± SD)

Group	*n*	IgM	IgG
Active UC group	36	0.43 ± 0.13[b]	0.61 ± 0.13[b,d]
Remittent UC group	20	0.38 ± 0.12[b]	0.50 ± 0.13[b]
Control group	25	0.20 ± 0.12	0.22 ± 0.14

[b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* remittent UC group.

Table 2 Positive expression rate for platelet activation marker in different groups of patients (%, mean ± SD)

Group	*n*	PAC-I	CD62P
Active UC group	36	30.6 ± 7.6[b,d]	45.0 ± 8.8[b,d]
Remittent UC group	20	19.6 ± 7.8[b]	31.9 ± 7.8[b]
Control group	25	6.3 ± 1.7	9.2 ± 2.7

[b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* remittent UC group.

chromatography, while molecular weight 50 000 and purity over 90% were determined by SDS-PAGE.

Detection of aβ2GP I in different groups of patients

Comparison of IgM aβ2GP I levels in different groups: there was a statistically significant difference in IgM aβ2GP I level between the active UC and control groups ($t = 7.010$, $P < 0.01$) and between the remittent UC and control groups ($t = 5.000$, $P < 0.01$). No statistically significant difference was found in IgM aβ2GP I level between the active and remittent UC groups ($t = 1.416$, $P > 0.05$).

Comparison of IgG aβ2GP I level in different groups: there was a statistically significant difference in IgG aβ2GP I level between the active UC and control groups ($t = 6.879$, $P < 0.01$) and between the active and remittent UC groups ($t = 3.034$, $P < 0.01$) (Table 1).

Positive expression rate for platelet activation marker in different groups of patients

The positive expression rates of platelet activation markers PAC-I and CD62P were sharply increased in the active UC group. There was a statistically significant difference between the active UC and control groups ($t_{PAC\text{-}I\ (\%)} = 15.679$, $t_{CD62P\ (\%)} = 19.664$, $P < 0.01$) and between the active and remittent UC groups ($t_{PAC\text{-}I\ (\%)} = 5.142$, $t_{CD62P\ (\%)} = 5.551$, $P < 0.01$). The positive expression rate in the remittent UC group was higher than that in the normal control group ($t_{PAC\text{-}I(\%)} = 8.305$, $t_{CD62P\ (\%)} = 13.306$, $P < 0.01$) (Table 2).

Correlation between aβ2GP I platelet activation marker and disease grades in 36 active UC patients

One-way ANOVA showed that there was a statistically significant difference of aβ2GP I level, PAC-I and CD62P in the disease grades (mild, moderate, severe) of patients with active UC [$F_{a\beta2GP\ I} = 3.679$ ($P < 0.05$), $F_{PAC\text{-}I\ (\%)} = 5.346$ ($P < 0.01$); F_{CD62P} (%) = 5.418 ($P < 0.01$, Table 3)]. Pearson correlation analysis displayed that the correlation coefficient of aβ2GP I (IgG) and PAC-I, and aβ2GP I (IgG) and CD62P PE was $r = 0.472$ and $r = 0.523$ ($P < 0.05$) respectively for mild active UC patients. The correlation coefficient of aβ2GP I (IgG) and PAC-I, and aβ2GP I (IgG) and CD62P was $r = 0.659$ ($P < 0.05$) and $r = 0.714$ ($P < 0.01$) respectively for moderate active UC patients. The correlation coefficient of aβ2GP I (IgG) and CD62P, and aβ2GP I (IgG) and PAC-I was $r = 0.714$ ($P < 0.01$) and $r = 0.878$ ($P < 0.01$) respectively for severe active UC patients. The correlation coefficient of aβ2GP I (IgG) and PAC-I, and aβ2GP I (IgG) and CD62P was $r = 0.878$ ($P < 0.01$) and $r = 0.902$ ($P < 0.01$) respectively for severe active UC patients.

Table 3 Correlation between aβ2GP I, platelet activation marker and disease grades in 36 active UC patients

Grade	*n*	aβ2GP I 492 (IgG)	PAC-I positive rate (%)	CD62P positive rate (%)
Mild	19	0.64	31.8	48.7
Moderate	11	0.80	40.6	60.9
Severe	6	0.92	53.7	71.2
F		3.679	5.346	5.418
P		< 0.05	< 0.01	< 0.01

DISCUSSION

β2GP I is a kind of glycoprotein in normal plasma. It has been shown in a recent study[19] that β2GP I plays an important role in the pathophysiological processes of human beings, such as production of APS autoantibody and formation of hypercoagulability state. Its molecule is a single polypeptide chain consisted of 326 amino acids and can be divided into five special functional domains under intermolecular disulfide bond influence, each of which is a circular permutation on the space. There are abundance amino acid sequences with positive charges in the fifth functional domain, the binding sites of which to phospholipids are located on C-terminal end. Cys281- Cys288 is the binding site and can combine with the surfaces of anionic phospholipids and particles with negative charges. One of their important physiological functions is to adjust the distribution of anionic phospholipids expressed on the activated platelets.

It was reported that immune factors play an important role in the pathogenesis of UC[25-27]. Thromboembolic disease is one of three main causes resulting in death of UC patients. Most scholars believe that hypercoagulability state, micro-thrombosis formation and platelet activation may play an important role in the pathogenesis of UC[28,29].

The pathogenesis of APS and UC mainly depends on immune factors, presence of hypercoagulability state and thrombosis formulation. aβ2GP I also plays an important role in the pathogenesis of APS[30].

In the present study, the serum aβ2GP I level in UC patients was measurable. The IgG aβ2GP I level in the active UC group was higher than that in the remittent UC and control groups, indicating that aβ2GP I may have a certain role in the pathogenesis of UC. Antiphospholipid antibody is just like one bridge between the two physiology systems, immunity and coagulation, which may lead to

arterial and venous thrombosis with unknown reason. Moreover, aβ2GP I is one of the most important autoantibodies. Whether aβ2GP I presence plays an important role in hypercoagulability state and thrombosis formation in ulcerative colitis remains unclear. We have proved, in this study, not only the platelet activation state but also the correlation between platelet activation and disease severity in active UC patients.

Flow cytometer and platelet glycoprotein McAb can accurately detect platelet activation. In this study, phycoerythrobilin labeled CD62P McAb was selected as a molecular marker, CD62P also known as GMP140, a protein on resting platelet α granule membrane. Since granules are released onto the platelet surface when platelets are in an active state, CD62P is expressed on the active platelet membrane instead of on the resting platelet membrane, which is a symbol of active platelets.

GPⅡb/Ⅲa is a glycoprotein on platelet membrane, which can be expressed in resting and active platelets, but only in activated platelets. The binding site of GPⅡb/Ⅲa and fibrinogen can be exposed. Moreover, since PAC-I is a McAb inhibiting the binding site, it is an effective means for detecting activated platelets because they can only combine with active platelets.

In the present study, the positive expression rate for platelet activation markers, PAC-I and CD62P, in active UC patients, was obviously higher than that in the control and remittent UC groups, indicating that platelet activation is one of the main reasons for blood hypercoagulability state in UC patients. At the same time, the positive expression rate for platelet activation markers in remittent UC group was higher than that in the control group ($P < 0.01$), indicating that platelet activation is a difficultly-eliminated factor. Anti-platelet activation therapy for patients at the remission stage can delay the disease progression and further improve prognosis and life quality of UC patients.

Not only high aβ2GP I level but also high activation state of platelets in patients with UC was detected in this study and the relationship between aβ2GP I level, platelet activation state and disease severity was further studied.

There was a significant difference in the positive expression rates of aβ2GP I (IgG), PAC-I and CD62P between the active UC patients, which increased when the disease was aggravated. Moreover, the positive expression rates of aβ2GP I (IgG), PAC-I and CD62P were positively correlated with the severity of the disease, suggesting that aβ2GP I and platelet activation play an important role in the development of ulcerative colitis and there is a mutual causality between aβ2GP I and platelet activation. Excessive inflammatory response may lead to loss of tolerance to some autoantigens. When the autoantibody is induced and damage is aggravated, inflammatory factors may cause platelet activation. Activated platelets and phospholipids with negative charges from the inner layer of cytomembrane are exposed on the surface of cytomembrane, which combines with β2GP I easily. After the combination, the concealed antigenic epitopes of β2GP I are exposed, thus producing anti-β2GP I antibody and other autoantibodies. aβ2GP I can not only combine with platelets but also activate platelets and endothelial cells[31], accelerating adhesion of monocytes and promoting the development of UC and complications, such as thrombosis.

In summary, aβ2GP I is one of the possible causes for blood hypercoagulability state in UC patients. The results of this study provide a new theoretical foundation for the pathogenesis of UC.

COMMENTS

Background

Although UC is an inflammatory bowel disease with unknown reasons, it is closely related to immunity and blood hypercoagulability state, just like APS. Anti-β2-glycoprotein antibody plays an important role in the pathogenesis of APS, which is our cut-in point in this study.

Research frontiers

The increased risk of thromboembolic complications in patients with UC is well recognized. It was reported that the incidence of thromboembolic events is 1%-8% in patients with inflammatory bowel disease. In addition, patients with inflammatory bowel disease may experience thromboembolic events at a younger age. Disease activity is a probable risk factor for thromboembolism. The hypercoagulable state in active UC patients is closely related to abnormal platelet function, resulting in a high probability of microvascular thrombosis and microcirculatory dysfunction, but so far, the reasons remain unclear.

Innovations and breakthroughs

Studies on factors such as vWF, XIIa, IX, VIIa, VIIIa, Va, and abnormal platelet function are available. In this study, we explored the cause for hypercoagulable state in UC patients based on the autoimmunity.

Applications

This study provided a new theory for the pathogenesis and development of UC, which is very helpful to estimate illness state and prognosis. Moreover, anti-thrombotic compounds seem to be therapeutically beneficial for UC patients.

Terminology

APS: Anti-phospholipid syndrome, a clinical condition characterized by recurrent thrombotic events and/or pregnancy morbidity associated with the persistence of antiphospholipid antibodies.

Peer review

This paper is very interesting because the authors studied an interesting problem related with the hypercoagulation state in some patients with ulcerative colitis.

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RAPID COMMUNICATION

Application of an indirect immunofluorescent staining method for detection of *Salmonella enteritidis* in paraffin slices and antigen location in infected duck tissues

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Abstract

AIM: To detect *Salmonella enteritidis* (*S. enteritidis*) in paraffin slices and antigen location in infected duck tissues.

METHODS: Rabbits were immunized with purified bacillus to obtain *S. enteritidis*-specific antibody, which were then extracted by the caprylic-ammonium sulphate method, purified through High-Q columns. An indirect immuno-fluorescent staining method (IFA) was established to detect the *S. enteritidis* antigen in paraffin slices. *S. enteritidis* was detected in each organ tissue of ducklings experimentally infected with *S. enteritidis*.

RESULTS: The gland of Garder, heart, kidney, spleen, liver, brain, ileum, jejunum, bursa of Fabricius from *S. enteritidis* experimentally infected ducklings were positive or strongly positive, and the *S. enteritidis* antigen was mainly distributed in the infected cell cytoplasm.

CONCLUSION: IFA is an intuitionist, sensitive and specific method in detecting *S. enteritidis* antigen in paraffin wax slices, and it is a good method in diagnosis and antigen location of *S. enteritidis*. We also conclude that the gland of Garder, heart, kidney, spleen, liver, ileum, jejunum are target organs in *S. enteritidis* infections of duck, and *S. enteritidis* is an intracellular parasitic bacterium.

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Key words: Indirect immuno-fluorescent antibody staining; *Salmonella enteritidis*; Paraffin tissues; Detection; Localization of antigens

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INTRODUCTION

Salmonella enteritidis (*S. enteritidis*) is the infectious disease causing Zoonoses. *S. enteritidis* is one of the primary causes of human food poisoning throughout the world, and has become a pointed public health problem[1-4]. China is the biggest country in the raising and consumption of duck in the world, but the *S. enteritidis* bacillus infection is a severely important infectious disease in the duck industry[5]. *S. enteritidis* outbreaks have been found to be associated with the consumption of contaminated and undercooked poultry products, such as eggs and egg-containing products, and have become a serious economic and public health problem[6]. Conventional methods for isolation of *S. enteritidis* are too laborious, not sufficiently sensitive or correct[7,8]. To our knowledge, a serum method for detection and antigen location of *S. enteritidis* has not been reported. Thus, to establish a rapid, sensitive and highly specific

method for detection of *S. enteritidis* is necessary. We established the Indirect Immunofluorescent (IFA) method to detect and antigen locate *Salmonella enteritidis* to offer a clinical means of diagnosis and apathogenic mechanism of ecology.

MATERIALS AND METHODS

Bacteria strain

S. enteritidis MY1 strain (obtained from Avain Disease Research Center of Sichuan Agriculture University).

Rabbit anti-serum preparation

Briefly, 5 mL of *S. enteritidis,* an overnight culture grown in LB broth, was harvested by centrifugation after 18 h at 37℃. Then diluted at 4×10^9 cfu and inactivated by formaldehyde and equaled to complete Freund's adjuvant (CFA) made the antigen. Rabbits were immunized with 1 mL antigen to obtain *S. enteritidis*-specific antibody, which was then extracted by the caprylic-ammonium sulphate method[9], purified through High-Q columns.

The establishment of the IFA

According to references[10], the following factors were considered to establish the method: (1) antigen recovery, to partly recover the antigens based on a microwave using 0.01 mol/L pH 6.0 citrate buffer solution for 20 min, 200 μg/mL parenzyme for 20min and no recovery; (2) rinsing water: 0.01 mol/L pH 7.4 PBS, 0.01 mol/L pH 7.4 PBS (containing 0.05% Tween-20) and rinsing water separately; (3) confining liquid: using 10% horse serum and 10% BSA to confine; (4) the diluted content of anti-rabbit-SE IgG: 1:25; 1:50; 1:100 and 1:200, incubate time: 4℃ overnight and 37℃ for 40 min; (5) the diluted content of the FITC labeled goat anti-rabbit IgG: 1:25; 1:50; 1:10 and 1:200; (6) foiled liquid: partly used 0.01% Evans Blue and unfoiled.

Artificial infected cases detection

Experimental infections were performed with a *S. enteritidis* MY1 strain. In brief, a group of 24 (specific antibody of SE negative) Bei Jing duck were inoculated with 0.2 mL (2×10^9 cfu) of *S. enteritidis* at the age of 7 d in the crop. Twenty-four uninoculated 7-d-old ducks were used as control animals.

After 24 h post-inoculation some of the ducks began to die resulting in organ harvesting. The organs were detected by IFA and conventional isolation respectively.

Treatment included first, using a non-selective pre-enrichment medium of buffered Peptone Water, incubated at 37℃ for 18 h. Then, using the selective enrichment media-Selenite Cystine Broth, incubating at 37℃ for 18 h. And then, transferring to SS agar, two differential media, Macconkey agar and Triple sugar iron agar were streaked and incubated at 37℃ for 24 h. Suspected colonies were picked up for biochemical and serological tests. The following tests were used: glucose, maltose, arabopyranose, mannitol, glycitol, hydrogen sulfide, MR, lysine decarboxylase, argininedecarboxylase, ornithinedecarboxylase, *etc.* Serotypes were identified by multivalence serum of $O_{A\text{-}F}$ and $O_{1, 9, 12}$ factor serum. The results were referred to[11,12].

The other organs were submerged in 4% formaldehyde, after 24 h; the organs were then embedded in paraffin. Then the tissue sections were made at 4 μm and stained with an indirect immunofluorescent technique.

Specific detection

The established method was applied to detect the livers from the dead ducks infected by *S. pullorum, S. gallinarum, E. coli Riemerella anatipestifer, P. multocida* and Duck plague virus (obtained from the Avian Disease Research Center of Sichuan Agriculture University).

The standard of judging results

The standard of the results are according to the presence, quantity, the depth and lighting of the coloured cells under the fluorescent microscope. There are no flavoviren cells judged negative, infra 5% weakly positive, 5%-50% positive, super 50% strong positive.

RESULTS

Results of anti-SE antibody

The purified anti-rabbit-SE IgG has an Antigen Jade Enlarge (AGE) potency to 1:32, through the SDS-PAGE, there are duplicate bands, and the molecular weight equals to the l-chain and H-chain of IgG. This suggests the IgG is highly pure.

The optimum conditions of IFA

The optimum conditions of this IFA were as follows: Ten minutes antigen retrieval by microwave with 0.01 mmol/L citrate buffer solution (pH 6.0); 10 min antigen retrieval by pancreatin; incubate in 10% bovine serum albumin at 37℃ for 30 min; dilute the primary antibody (1:25) and incubate for 40 min at 37℃; and then incubated at 37℃ for 30 min with diluted FITC-labeled-secondary antibody (1:100) which contains 0.01% Evans.

Distribution in organs of the infected ducks

We detected the organs by IFA after 24 h, a large part of the organs had positive results, and the results were as follows (Figure 1): Heart: Presented as a strong positive, Cardiac muscle fiber had positive cells, and the positive signal distributing the cytoplasm (Figure 1A). Kidney: Presented positive, the mesenchyme between the tube of the Kidney had positive cells, and the positive signal distributing the cytoplasm (Figure 1B). Liver: Presented positive, the mesenchyme between the hepatic cord had positive cells, and the positive signal distributing the cytoplasm (Figure 1C). Bursa of Fabricius: presented positive, the area of the medulla of the follicle had positive cells, and the positive signal distributing the cytoplasm (Figure 1D). Brain: Presented positive, positive cells presented in capillary vessels, and the positive signal distributing the cytoplasm (Figure 1E). Gland of Garder: Presented as a strong positive, the mesenchyme of the tube of the gland had positive cells, and the positive signal

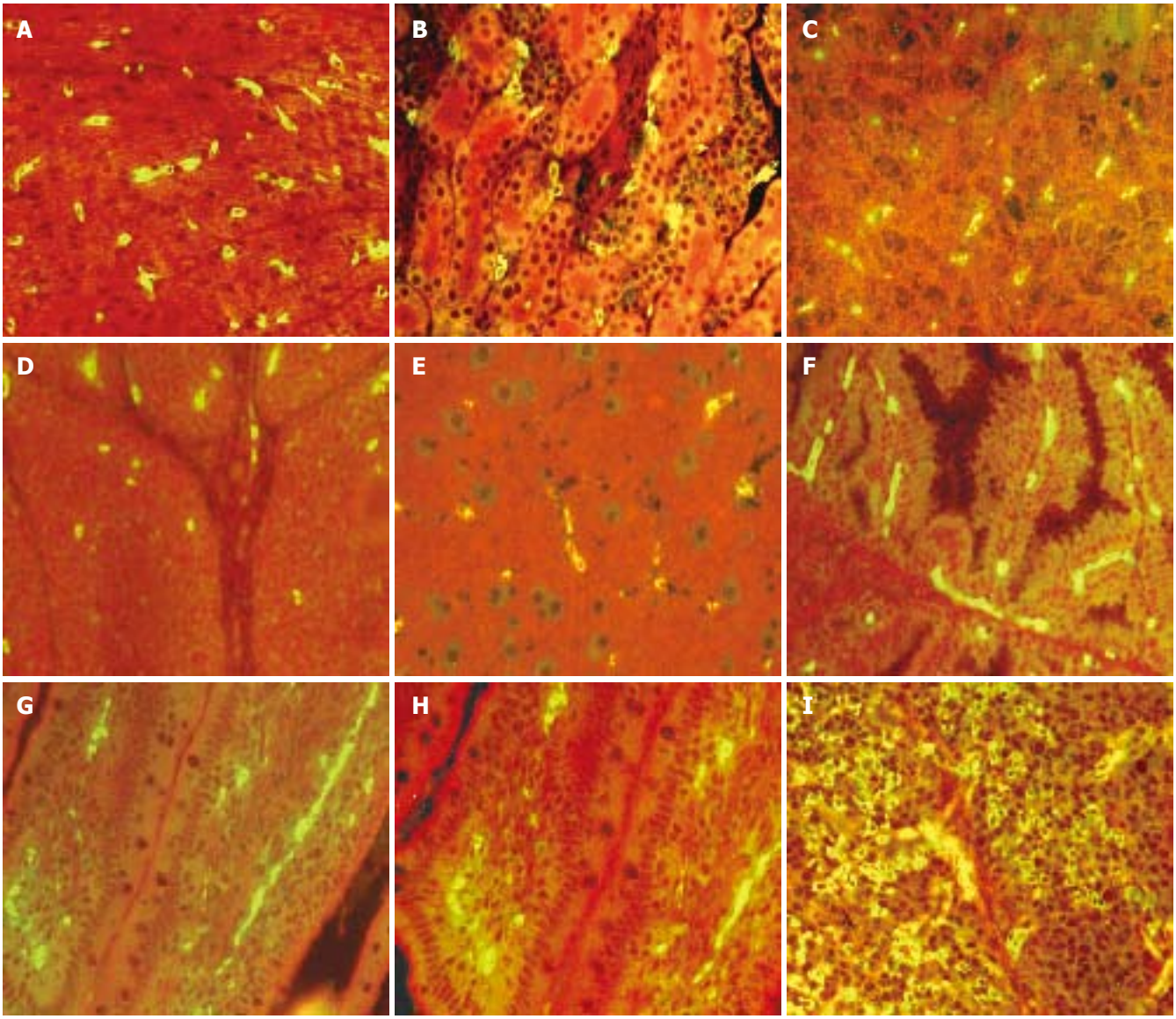


Figure 1 Organ tissues from SE-inoculated ducks immunofluorescent stain for SE. **A**: Positive staining baclli are adhering to the Cardiac muscle fiber (Heart); **B**: Positive staining baclli are adhering to the mesenchyme between the tube of Kidney (Kidney); **C**: Positive staining baclli are adhering to the mesenchyme between the Hepatic cord; **D**: Positive staining baclli are adhering to the area of medulla of the Follicle; **E**: Positive staining baclli are adhering to vascular endothelial cell; **F**: Positive staining baclli are adhering to the mesenchyme of the tube of the gland; **G, H**: Positive staining bacllis are adhering to the interstitial tissue of the lamina propria in villi; **I**: Positive staining baclli are adhering to red medulla. (Images were acquired by using 40 x objective).

Table 1 The results of man-made clinical cases detection using isolation and IFA methods

Detection methods	Results of different organs (faeces) by different methods (positive/total)								
	Heart	Liver	Spleen	Lung	Kidney	Intestine	Brain	Thymus	BF
Isolation	2/24	24/24	24/24	2/24	24/24	24/24	3/24	0/24	8/24
IFA	24/24	24/24	22/24	0/24	24/24	20/24	2/24	0/24	5/24

distributing the cytoplasm (Figure 1F). Small intestine: Numerous *S. enteritidis* organs were observed lying free within the interstitial tissue of the lamina propria in the villi, and the strong positive signals observed in the cytoplasm (Figure 1G, H). Spleen: Presented strong positive, a number of positive cells with particle immunofluorescent antigen distributed in the red medulla of the spleen (Figure 1I).

The results of conventional isolation

All the positive samples detected by the isolation method can be detected by this IFA (Table 1).

The results of specific detection

We detected the livers from the dead ducks infected by *S. pullorum, S. gallinarum, E. coli Riemerella anatipestifer, P.multocida* and Duck plague virus, *S. enteritidis*. The result

Table 2 The results of the specific detection, the positive labeled with (+), negative (-)

Strains	*S. pullorum*	*S. gallinarum*	*E. coli*	*R.A*	*P.multocida*	*DPV*	*SE*
Results	-	-	-	-	-	-	+

showed only the *S. enteritidis* infected, present positive reaction; the others have negative reaction. This suggested good establishment for specificity of the IFA (Table 2).

DISCUSSION

Comparing the differences between IFA and other methods

In the present study, there is little to report about the clinical diagnosis beside conventional methods of isolation for *S. enteritidis*, but there is much about other species. Gast *et al*[6] collected various organs of the infected birds and isolated the bacteria, but it is too laborious, not sufficiently sensitive and correct and can not be used with the rapid diagnoses. Kim *et al*[13] detected the *S. enteritidis* by enzyme immunoassay and had a good result. Desmidt *et al*[14] observed the *S. enteritidis* in the ceca of chickens by immunohistochemistry, which can be used to locate sub-cells of organ tissues of *S. enteritidis* infected chickens. Coope[15] detected the *S. enteritidis* by Dot-Blot and Western-Blot, which is rapid and specific, but could not detect the location in sub-cells of organ tissues. Compared to the above method, the IFA method established has the ability to detect and locate cells and organ tissues of *S. enteritidis* infection, thus offering a clinical means of diagnosis and a pathogenic mechanism of ecology.

The distribution of S. enteritidis in organ tissues

We checked all the ducks when they began to die. At 24 h post-inoculation, the positive signals were observed in the small intestine and spleen; at 36 h, in the heart, kidney, livers, bursa of Fabricius, and Gland of Garder; at 72 h, the positive signals were at the peak; at 6 d post-inoculation, the signals began to reduce. From the result (Figure 1) of the study, we know that the IFA can detect the antigens in almost all organ tissues. The small intestine (ileum, jejunum), heart, liver, kidney, spleen, the Gland of Garder have strong positive signals (Figure 1A-C, I), and presented in the cytoplasm of phagocytic cells of the macrophile system. This may be caused by phagocytic cells after entering the host. Meanwhile, we can detect a positive signal in the brain, a positive signal presented in the vascular endothelial cell, which may be caused by *S. enteritidis* invading the organs and *S. enteritidis* in blood. At the anaphase of infection, the infection appeared bacteremic, the *S. enteritidis* needed to transit the vascular endothelial cells, and uniformly distributed in the cytoplasm of the vascular endothelial cells. We also detected small positive signals in the bursa of Fabricius.

The value of S. enteritidis infections from other viral infectious diseases

Immuno-fluorescent labeled bacilli were associated with the epithelial surface of the intestinal villi at 4 h. At 24 a strong positive signal was detected intracellularly in the small intestine (Figure 1G, H). This resulted in a positive signal which only presented in the lamina propria of the villi. This is a big difference from Duck plague virus (DPV)[16] and Duck Hepatitis Virus (DHV)[17], which was located in the lamina propria. This variation might be related to a different microenvironment[18] in different parts of the gut, dividing into a microcolony at that site. The microenvironment has an important effect in the colonization of the gut, primarly as follows. Gastric acid is the first defense of the intestinal infection, the bacterium can hardly survive (pH < 1.5). The slime layer of the intestinal mucosa has a barrier function to the *S. enteritidis*; moreover pankrin, bile salt and small intestinal juice can depress the bacterium. Some studies suggest that the gene encoding the invasive protein is depressed because of the bile[19,20]. Meanwhile, the involvement of mucosal IgA in the protection against *S. enteritidis* involving the epithelial surface has been reported[21,22]. The cathodolyte antibacterial peptide secreted by pit cells in the small intestine belongs to the Toalexin family, which can increase the permeability of the cytomembrane of the bacterium, and not profit to survive[23].

The intestine is an important barrier for prevention against invasion by bacteria. The lymphoid tissue called Peyer macular under the muscoa[24] consists of T cells, B cells, macrophages and dendritic cells, following the swallowing of the pathogenic bacterium.

The immune reaction also played an important role in preventing *S. enteritidis* lying free in the endothelial cells of the intestine. After swallowing the *S. enteritidis* by macrophage and heterophil granulocyte, both happened to outbreak by breathing, which produced oxygen free radical, for instance, O_2-, H_2O_2, OH-, and O_2- and so on. The oxygen free radical can kill and wound bacteria; the cell factors (IL-1, IL-6, TNF-α[25]) composed and released have a pathopoiesis effect and a bactericidal effect; when the *S. enteritidis* invades the enterocyte, it can induce the secretion of IL-8[26], which can kill and wound bacteria. $CD4^+$ T cells also have an effect on the immune defense[27].

The cooperation between the host cells and S. enteritidis infections

Infection with bacteria is usually started by oral ingestion of the pathogen and is followed by bacterial colonization of the gut and invasion of internal tissues. Intracellular replication is essential for the virulence.

Attachment to host tissues is the first important step to establish a bacterial infection. The attachment is primarily referred to as the inv gene[28]. Once attached to the host cells, the bacterium manifests athletic phenomenon of invasion of the cells, phagocytosis by cell invagination. After fusion of macrophage lysosomes with phagosomes, the *S. enteritidis* appears in the phagocytotic vesicle of the cytoplasm[29], and may destroy the cell invagination. In the present study, there are different reports about the *S. enteritidis* entering the cell, the bulk of the investigations

support the view that *S. enteritidis* enters the cell by a virulence factor. Higashide and McGhie suggest that when *S. enteritidis* enter the cell, firstly SspC excreted by TTSS insert into the plasma membrane, then Sip and SipA bind to the actin of the cytoskeleton and rearrange the frame, which enhances the entrance of the bacterium[30,31]. Scientists report recently that the dendritic cell (DC) residing in the mucosa of the small intestine can use the dendrite to open the tight junction of the cells, and directly ingest the bacteria from the mucosa[32]. From the result (Figure 1), the positive signal distributed in the cytoplasm of infected cells of the organ tissues, we conclude that *S. enteritidis* is an intracellular parasitic bacterium.

COMMENTS

Background

Salmonella enteritidis (*S. enteritidis*) is the infectious disease causing Zoonoses. *S. enteritidis* is one of the primary causes of human food poisoning throughout the world. China is the biggest country in growing and consumption in the world, but the *S. enteritidis* bacillus infection is the severely important infectious disease in raising duck's industry. But the pathogenic mechanism of ecology is unknown.

Research frontiers

The *S. enteritidis* can be isolated from various organs of the infected birds. It is possible that the *S. enteritidis* invades the tissues of these organs; electron microscopic studies of chicks have shown passage of *Salmonella* through ileocecal mucosa. The observation of attachment and invasion of the ceca of infected chicks have been reported. Also, in mammalian animal models, the colonization of *S. enteritidis* has been studied further. However, there are few studies about ducks.

Innovations and breakthroughs

The present study was to establish a serum method for detection and antigen location of *S. enteritidis* which has not been reported.

Applications

The results can offer a clinic means of diagnosis and pathogenic mechanism of ecology.

Peer review

This is a methodology manuscript. The author of this manuscript establishes a method of IFA and detected the organs of the *S. enteritidis* infected ducks. The author conclude that IFA is a sensitive and specific method in detecting *S. enteritidis* antigen in paraffin slice; it's a good method in diagnosis and antigen location of S. enteritidis; the Gland of Garder, heart, kidney, spleen, liver, and small intestine are target organs in *S. enteritidis* infections of duck; *S. enteritidis* is an intracellular parasitic bacterium.

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RAPID COMMUNICATION

Quantitative studies of the regular distribution pattern for *Salmonella enteritidis* in the internal organs of mice after oral challenge by a specific real-time polymerase chain reaction

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Abstract

AIM: To identify and understand the regular distribution pattern for *Salmonella enteritidis* (*S. enteritidis*) in the internal organs of mice after an oral challenge over a 3 wk period.

METHODS: Assays based on the serovar-specific DNA sequence of *S. enteritidis* from GenBank, and a serovar-specific real-time, fluorescence-based quantitative polymerase chain reaction (FQ-PCR) were developed for the detection of *S. enteritidis*. We used this assay to detect genomic DNA of *S. enteritidis* in the blood and the internal organs, including heart, liver, spleen, kidney, pancreas, and gallbladder, from mice after oral challenge at different time points respectively.

RESULTS: The results showed that the spleen was positive at 12 h post inoculation (PI), and the blood was at 14 h PI. The organism was detected in the liver and heart at 16 h PI, the pancreas was positive at 20 h PI, and the final organs to show positive results were the kidney and gallbladder at 22 h PI. The copy number of *S. enteritidis* DNA in each tissue reached a peak at 24-36 h PI, with the liver and spleen containing high concentrations of *S. enteritidis*, whereas the blood, heart, kidney, pancreas, and gallbladder had low concentrations. *S. enteritidis* populations began to decrease and were not detectable at 3 d PI, but were still present up to 12 d PI in the gallbladder, 2 wk for the liver, and 3 wk for the spleen without causing apparent symptoms.

CONCLUSION: The results provided significant data for understanding the life cycle of *S. enteritidis* in the internal organs, and showed that the liver and spleen may be the primary sites for setting itself up as a commensal over a long time after oral challenge. Interestingly, it may be the first time reported that the gallbladder is a site of carriage for *S. enteritidis* over a 12 d period. This study will help to understand the mechanisms of action of *S. enteritidis* infection *in vivo*.

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Key words: Fluorescence-based quantitative polymerase chain reaction; Internal organs; *Salmonella enteritidis*; Regular distribution pattern

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INTRODUCTION

A significant proportion of human salmonellosis is caused

by consumption of raw or partially cooked eggs[1-3]. *Salmonella enteritidis* (*S. enteritidis*) is one of the main causes of gastrointestinal infection in China. Incidence of infection is highest in children, elderly, and immunosuppressed individuals[4]. *S. enteritidis* is a facultative intracellular pathogen capable of causing disease in a wide range of host species. After oral ingestion, *S. enteritidis* cells rapidly reach the bowel and penetrate the macrophages, spread to the mesenteric lymph nodes, and in severe cases, can reach the circulatory system[5]. The macrophages carry the organism to the liver, pancreas, and spleen, where the bacteria are thought to replicate in both phagocytic and nonphagocytic cells[5]. Also, it was reported that the *S.* typhimurium can be shed into the gallbladder from the liver, where either an active infection (cholecystitis) or a chronic infection can develop[6,7].

Previous studies showed that *S. enteritidis* was usually cleared from internal organs more than 8 wk after inoculation of poultry. How *S. enteritidis* survives within macrophages is unclear. It seems likely that the type Ⅲ secretion system encoded by *Salmonella* pathogenicity island 2 may play a major role. Moreover, extensive studies of mouse have shown the role for T cells, natural killer cells, macrophages, neutrophils and numerous cytokines in *Salmonella* resistance[8,9]. Numerous studies indicate that *S. enteritidis* cells have evolved strategies to resist and overcome innate immune defenses[10,11]. Up to date, the mechanisms by which *S. enteritidis* and other serotypes persist within the host and the reasons for the absence of immune clearance are not known. As a result of the increased prevalence of *S. enteritidis* and its complex life cycle, identifying the regular distribution pattern of *S. enteritidis* in the internal organs over a 3 wk period, which is not described hitherto, will help to understand its mechanism of action.

One of the main advantages of FQ-PCR is the ability to quantitate unknown samples. With this assay, it is possible to carry out a rapid quantitative analysis of DNA over a wide linear range, with an unknown template. The specific DNA fragment (Sdf I) of *S. enteritidis* was reported by Agron *et al*[12], which was screened for using the Supression Subtractive Hybridization method, and appears to only be found in serovar *enteritidis* strains[12,13]. Here, based on the study of Agron *et al*, we developed a standard curve (this methodology is the first time established which based upon the Sdf I DNA fragment), and then applied this to the study of internal organ distribution of *S. enteritidis* in mice.

MATERIALS AND METHODS

Bacterial strains

A total of 19 *Salmonella* strains were included in this study. Most strains were purchased from the National Center for Medical Culture Collection, including *S. enteritidis* (Human, No. 50041), *S. enteritidis* (Human, No. 50040), *S. enteritidis* (Mouse, No. 50338), *S. enteritidis* (Human No. 50100), *S. enteritidis* (Human, No.50128), *S. enteritidis* (Human, No. 50335), *S. enteritidis* (Mouse, No. 50336), *S. enteritidis* (Human, No. 50760), *S. choleraesuis* (No. 50191-1), *S. typhi* (No. 50013), *S. typhimurium* (No. 50115-13), *S. paratyphi* (No. 50001-24), *S. pullorum* (No. 50047-2), *S. anatum* (No. 50083-4), *S. gallinarum* (No. 50770), *S. dublin* (No. 50761). Three strains were isolated and maintained by the Avian Diseases Research Center, Collage of Veterinary Medicine of Sichuan Agricultural University, including *S. enteritidis* (Duck, No. MY_1), *S. enteritidis* (Duck, No. SC1), *S. enteritidis* (Chicken, No. CD1).

Preparation of bacterial samples and generation of standard templates

Briefly, 5 mL of bacterial culture was grown overnight in Luria-Bertani broth and 500 μL of the culture was harvested by centrifugation. The pellet was resuspended in 500 μL TE buffer (pH 8.0) and 2 μL of lysozyme solution (30 g/L) was added, followed by lysis using 10% SDS (80 μL) at 60℃ for 1 h. DNA was purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Then, a 1/10 volume of 3 mol/L sodium acetate and 2 volumes absolute ethanol were added, and the nucleic acid was then pelleted by centrifugation, washed with 70% ethanol, and dried under vacuum. The DNA genomic pellet was resuspended in 30 μL TE buffer (pH 8.0), and stored at -20℃ until use.

A conventional PCR was carried out using a template from *S. enteritidis* (Chicken, No. CD1), with primers F_1 and R_1 (designed with Sdf I, Genbank Accession No. AF370707.1, generated by TakaRa Biotech, DaLian, China). The primer sequences from 5' to 3', were as follows: F_1, TGTGTTTTATCTGATGCAAGAGG; and R_1, CGTTCTTCTGGTACTTACGATGAC. Amplification was carried out in a total volume of 50 μL, containing 1 μL each primer (25 μmol/L), 1 μL dNTPs (10 mmol/L), 2.5 U Taq DNA Polymerase (TaKaRa Taq, TakaRa Biotech), 5 μL 10 × PCR buffer (with Mg^{2+}, 25 mmol/L), and 6 μL templates, then made up to a volume of 50 μL with deionized water. An initial denaturation at 95℃ for 5 min was followed by 32 cycles of denaturation at 94℃ for 30 s, annealing at 52.5℃ for 30 s, and extension at 72℃ for 40 s. Finally, an additional extension was achieved for 10 min at 72℃. The product size was 293 bp.

Finally, the product was gel-excised and quantified with appropriate standards. Its concentration was determined spectrophotometrically using the Bio-Rad-Smartspec-3000 instrument, according to the manufacturer's instructions. The standards were diluted, divided into aliquots, and frozen before used.

Development of FQ-PCR and its products

The FQ-PCR assay, including volume, Mg^{2+} concentration, probe and primer concentrations, and annealing temperature were optimized initially. Subsequently, the sensitivity of the assay, the linear range and standard curve were determined by using known amounts of purified template DNA (generated as described above). The primers (F_2 and R_2) and TaqMan-probe (FP) of FQ-PCR were designed using an internal region of the 293 bp sequences (described above, generated by TakaRa Biotech), and were used as follows, from 5' to 3': F_2, TTGATGTGGTTGGTTCGTCACT; R_2, TCCCTGAA TCTGAGAAAGAAAAACTC; and TaqMan-probe (FP), FAM- TGCAGCGAGCATGTTCTGGAAAGC-TAMRA.

Amplification of FQ-PCR was carried out in a total volume of 25 μL, containing 0.6 μL each primer (10 μmol/L), 0.75 μL dNTPs (10 mmol/L), 1.25 U EX Taq DNA Polymerase (TaKaRa EX Taq Hot Start Version, TakaRa Biotech), 5 μL 5 × PCR buffer (free Mg^{2+}), 0.8 μL TaqMan-probe (5 μmol/L), 0.5 μL Mg^{2+} (250 mmol/L) and 5 μL aliquot of the sample DNA templates (for the standards DNA templates, 1 μL was added), then made up to a volume of 25 μL with deionized water. Each run consisted of a 95℃ 5 min hot start, which activated the conjugated polymerase, followed by 45 cycles with a 94℃ denaturation for 30 s, 55℃ annealing for 30 s, and reading the fluorescent signal at this step.

The primers of FQ-PCR (F_2 and R_2) were used for conventional PCR with *S. enteritidis* (Chicken, No. CD1) DNA templates, in order to verify the specific amplification. Amplification was carried out in volume of 50 μL, containing 1 μL each primer (25 μmol/L), 1 μL dNTPs (10 mmol/L), 2.5 U Taq DNA polymerase (TaKaRa Taq, TakaRa Biotech), 5 μL 10 × PCR buffer (with Mg^{2+}, 25 mmol/L), and 6 μL templates, then made up to a volume of 50 μL with deionized water. An initial denaturation at 95℃ for 5 min was followed by 32 cycles of denaturation at 94℃ for 30 s, annealing at 49℃ for 30 s, and extension at 72℃ for 40 s. Finally, an additional extension was achieved for 10 min at 72℃. A 10 μL aliquot of PCR product was electrophoresed on a 1.5% agarose gel for 40-50 min at 80 V, and visualized and photographed under UV illumination. Simultaneously, DNA sequences of the products were carried out by TakaRa Biotech.

FQ-PCR standard curve

Based on the previous studies to generate the standard curve as follows[14-16]: Standards DNA were used to establish a standard curve and the standards contained amplified target DNA in different quantities which were measured by fluorimetic analysis (iCyclerQ, Bio-Rad, USA). The Primers F_2 and R_2 were used for this amplification, the DNA was 10-fold serially diluted in nuclease-free water and standard curve was generated by using 1.0×10^5 to 1.0×10^{10} gene copies of standards DNA. Concentrations of the standards were measured by fluorimetic analysis, then an analysis of key cycler measurements were performed after each run to verify identical amplification efficiencies and conditions between runs. Finally, based on the data generated, a standard curve for the icycler was obtained and based on the standard curve to obtain the copies number of *S. enteritidis* for the samples.

Specificity,sensitivity and reproducibility of the FQ-PCR

All 19 bacterial strains were used to assess the specificity of the FQ-PCR. The phenol/chloroform/isoamyl alcohol method (described above) was used to prepare the DNA template, 6 μL of this aliquot was used in FQ-PCR.

To determine the detection limit of this FQ-PCR assay, different quantities of standard DNA of *S. enteritidis* was added. We used phenol/chloroform/isoamyl alcohol method to extract DNA of tissue from several control group samples (described below), and added 1.0×10^5-1.0×10^{-1} copies of the standard DNA for each. Finally the results were measured by fluorimetric analysis.

To evaluate the variability between experiments, three different known concentrations of DNA were amplified by performing the assay described above in triplicate. For each experiment, the crossing point, average crossing point, standard deviation, and coefficient of variation for each assay were calculated.

Experimental infection of mice

Our infection model was based on the previous studies, which showed that orally introduced *S. enteritidis* had a rapid transit time through the intestine and established itself within the walls of the gut in more than 3 d[17,18]. 80 mice (age 9 wk, specific-pathogen-free) were purchased from the Animal Center of Sichuan University, China. In brief, a group of 60 mice was oral infected with a virulent *S. enteritidis* strain (Chicken, No. CD1, LD_{50} is 4.0×10^8 cells after oral challenge), at 4.0×10^6 cells per mouse (In our preliminary experiments, the results showed that this dose of challenge can induce clinical signs and pathology in mice, but can not cause death). Another group of 20 mice was treated with an equal volume of water as a control. Blood, heart, liver, spleen, kidney, pancreas, and gallbladder were analyzed by FQ-PCR at different post-inoculation time points, at 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 14 h, 16 h, 18 h, 20 h, 22 h, 24 h and 36 h and 2 d, 3 d, 6 d,9 d,12 d and 2 wk,3 wk.

Three mice from infected group and one from control group were sacrificed at each time point (There was a potential complication of *S. enteritidis* DNA in the blood for the tissue results, particularly in the spleen and liver where large amounts of blood could be accumulated, so the mice were perfused before the tissue collection) and its organs were aseptically harvested and immediately placed in 1.5 mL labeled snap-cap tubes, and frozen. The blood sample, 200 μL, was obtained from the vena caudalis with syringe aseptically before sacrificed. For the bile sample, 200 μL was obtained from gallbladder with syringe aseptically after sacrificed, and frozen.

DNA extraction from tissue samples was described below: Briefly, 0.5 g of the tissue sample was ground up using a tissue grinder in the 1.5 mL Eppendorf tube. The pellet was resuspended in 500 μL TE buffer (pH 8.0) with 10 μL Proteinase K (30 g/L) and incubated at 37℃ for 2 h. Finally, with a conventional phenol/chloroform/isoamyl alcohol method (described above), to extract the genomic DNA of *S. enteritidis* from tissue, used 5 μL aliquot of DNA template for FQ-PCR detection.

For the bile and blood samples, taking 200 μL to be harvested by centrifugation, then the pellet was resuspended in 500 μL TE buffer (pH 8.0) with 3 μL Proteinase K (30 g/L), and incubated at 37℃ for 1 h. Finally, used the phenol/chloroform/isoamyl alcohol method (described above) to extract DNA template and used 5 μL to be detected.

Comparative the differences between FQ-PCR and the traditional bacterial culture method

All the samples of mice were detected by the traditional bacterial culture method in order to identify the accuracy of this FQ-PCR method. Firstly, taking 0.1 g or 20 μL tissue sample, then used the selective enrichment media-

Selenite Cystine Broth, incubated at 37℃ for 20 h. And then, transferred to *Salmonella* Shigella Agar (SS agar). two differential media, Macconkey agar and Triple sugar iron agar were streaked and incubated at 37℃ for 24 h. Suspected colonies were picked up for biochemical and serological tests. The following tests were used glucose, maltose, arabopyranose, mannitol, glycitol, Hydrogen sulfide, MR, lysine decarboxylase, argininedecarboxylase, orinithinedecarboxylase, *et al.*

Serotypes were identified by multivalence serum of O_{A-F} and $O_{1,9,12}$ factor serum. The results were referred to[19,20] incomplete sentence-authors must complete.

Statistical analysis

All samples were analyzed three times with a mean and standard error. Data were analyzed on an IBM compatible personal computer using SPSS version 11.0. Effects were considered to be significant if $P < 0.05$. Finally, based on the standard curve (described aboved) to obtain the copies number of *S. enteritidis*. The *S. enteritidis* DNA copy concentrations were expressed as the mean $\log_{10}$ copies genome numbers per 0.5 g or 0.2 mL of tested tissue or blood and bile, respectively.

RESULTS

Specific verification of FQ-PCR products

The primers of FQ-PCR were used for conventional PCR with *S. enteritidis* (Chicken, No. CD1) DNA templates, in order to verify the specific amplification. Results showed that the PCR produced an intense band with the expected 130 bp for *S. enteritidis*, which indicated 100% specificity. Also, Sequence analysis was carried out using BLASTn and BLASTn Programs of National Center for Biotechnology Information, and resulted in 100% homology with DNA of *S. enteritidis*, Genbank Accession No.AF370707.1.

FQ-PCR standard curve

By using standards template containing from 1.0×10^5 to 1.0×10^{10} copies, accurate results for a series of samples were obtained, based on the data used to generate the standard curve with the iCycler IQ Detection System (Bio-Rad; USA). The correlation coefficient for the associated standard curve was 1.000 and PCR efficiency was 98.2%, which indicated that the crossing threshold values for the standards fell within accurate range. Through the formula as follows, we could quantitate the DNA copies of *S. enteritidis* for unknown samples, Y= -3.366X + 44.914 (where Y is the threshold cycle, and X is the log of the starting quantity) (Figure 1).

Specificity, sensitivity and reproducibility of the FQ-PCR

All 19 bacterial strains were used to assess the specificity of the FQ-PCR, indicated that only *S. enteritidis* strains genomic showed the positive results, while there was no positive results with none *S. enteritidis* strains.

A range from 1.0×10^5 to 1.0×10^{-1} copies of *S. enteritidis* standards template was used, the limit of detection was 10 copies/μL.

Three different, known concentrations of DNA, 1.0×10^9 to 1.0×10^7 copies/μL, were amplified by performing the assay described above in triplicate. Analysis of these values proved that the assay was reproducible, as the coefficient of variation was statistically low, at < 1.8%, the standard deviation was 1.1%, and the threshold cycle for each concentration was difference between 0.2 cycle and 0.4 cycle, highly reproducible.

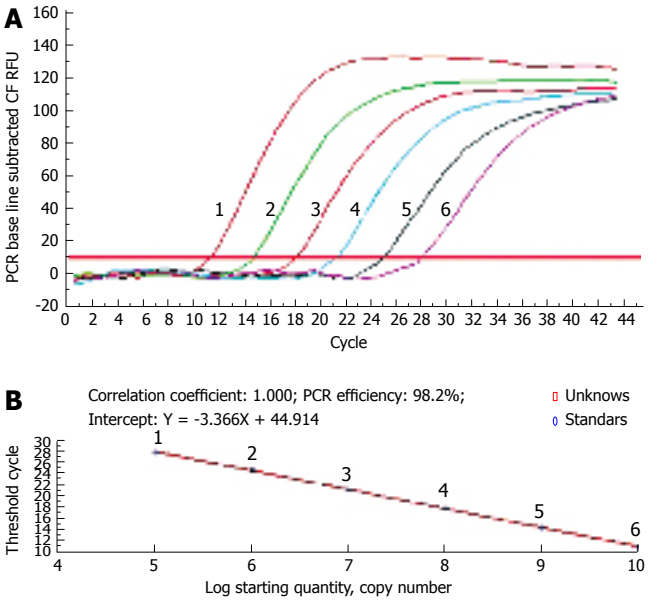


Figure 1 Used the standards DNA template with 10-fold serial dilutions to develop standard curve. **A**: Amplification curve; **B**: Standard curve; 1, 1.0×10^{10} copies/μL; 2, 1.0×10^9 copies/μL; 3, 1.0×10^8 copies/μL; 4, 1.0×10^7 copies/μL; 5, 1.0×10^6 copies/μL; 6, 1.0×10^5 copies/μL.

Clinical signs and gross necropsy

S. enteritidis-inoculated mice appeared to be clinically normal, and there were no sings of depression or diarrhea, and feeding and drinking behavior remained normal at 30 min to 8 h, and 6 d to 3 wk PI. However, mice developed significant clinical signs of *S. enteritidis* infection at 12 h to 3 d PI. At necropsy, gross lesions were observed in all of the mice at this period, such as hyperemia of intestine, swelling of gallbladder[19,20].

Distribution of S. enteritidis in the internal organs

The distribution of *S. enteritidis* within the internal organs after oral challenge was determined by means of FQ-PCR, over a 3 wk period at intervals. Results showed that the spleen was positive at 12 h PI, with about 2.95×10^2 copies/0.5 g. Then, blood had positive results at 14 h PI with about 2.75×10^2 copies/0.2 mL, and the organism was detected in the liver and heart at 16 h PI, pancreas was positive at 20 h PI, and the last organ to show a positive results were the kidney and gallbladder at 22 h PI. The copy numbers of *S. enteritidis* in each tissue reached a peak at 24-36 h PI, with the liver, spleen containing high concentrations of *S. enteritidis*, about 2.00×10^4 to 3.31×10^6 copies/0.5 g, whereas the blood, heart, kidney, pancreas and gallbladder had low concentrations, ranked from 5.13×10^2 to 1.70×10^5 copies/0.5 g (0.2 mL). Numbers of bacteria decreased at 3 d-9 d, the level of *S.*

Table 1 The distribution and quantity of *S. enteritidis* within different internal organs and blood after oral challenge at different times

Time	Blood	Heart	Liver	Spleen	Kidney	Pancreas	Gallbladder
30 min	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12 h	0.00	0.00	0.00	2.47	0.00	0.00	0.00
14 h	2.44	0.00	0.00	2.80	0.00	0.00	0.00
16 h	2.68	2.72	2.83	3.22	0.00	0.00	0.00
18 h	2.91	3.21	3.31	3.61	0.00	0.00	0.00
20 h	3.41	3.46	3.76	3.85	0.00	2.43	0.00
22 h	3.75	3.62	3.93	4.20	2.50	2.88	2.44
24 h	3.94	3.97	4.30	4.36	2.80	3.12	2.82
36 h	4.11	5.28	6.46	6.52	4.46	4.16	2.71
2 d	3.78	3.01	4.11	5.59	2.75	2.82	2.60
3 d	2.50	2.81	3.80	5.22	0.00	2.27	2.52
6 d	0.00	0.00	3.02	4.02	0.00	0.00	2.36
9 d	0.00	0.00	2.69	3.78	0.00	0.00	2.22
12 d	0.00	0.00	2.32	3.52	0.00	0.00	2.10
2 wk	0.00	0.00	2.10	2.82	0.00	0.00	0.00
3 wk	0.00	0.00	0.00	2.48	0.00	0.00	0.00

$\log_{10}$ copies per 0.2 mL for Blood and bile; $\log_{10}$ copies per 0.5 g for others.

enteritidis clearly decreased, with the blood, heart, kidney, and pancreas not having a positive result. The mice were capable of carrying *S. enteritidis* cells were present up to 12 d PI in the gallbladder, 2 wk for the liver, and 3 wk for the spleen without causing any apparent symptoms. Importantly, the level of *S. enteritidis* cells number compared to the other organs at 2-12 d PI, the gallbladder can contain about 1.25×10^2 to 7.94×10^2 copies/0.2 mL. It may be the first time reported that the gallbladder is a site of carriage for *S. enteritidis*, and *S. enteritidis* can persist over a 12 d period within it. Also, the control group did not have any positive results at any time in any location. The details are shown in Table 1.

The results of the traditional bacterial culture method

All the samples from different time points were detected by traditional bacterial culture method, and the results showed that the traditional bacterial culture method had positive results when the *S. enteritidis* target DNA concentrations were > $10^{4.02}$ copies per 0.5 g or 0.2 mL. Apparently, the FQ-PCR assay provides a more sensitive and accurate method for the this study.

DISCUSSION

Once attached to the host cells, *S. enteritidis* can manifest athletic phenomenon of invasion, such as phagocytized by cell invagination. After fused by macrophage, *S. enteritidis* cells will appear in cytoplasm, survive and multiply there[21]. Humoral immunity contributes to serve as the first line of defense against *S. enteritidis* infection. After the *S. enteritidis* cells are swallowed by macrophage and heterophil granulocyte, both of them happen to outbreak of breathing and produce oxygen free radical, for instance H_2O_2, OH-, O_2^-[22], which can kill and wound the bacterium. The cell factors, such as IL-1, IL-6 *et al*, also have the bactericidal effect for the *S. enteritidis* invasion[23]. In contrast, cellular immune responses have been rarely investigated, although such responses are crucial for protective immunity against *S. enteritidis*. The mechanisms by which *Salmonella*-specific $CD4^+$ T cells contribute to protective immunity are incompletely understood, but T-cell proliferation, the sine qua non of $CD4^+$ T-cell activation, and the production of gamma interferon can be regarded as in vitro indicators of these essential elements of protective immunity[24]. Our data (Table 1) showed that the *S. enteritidis* populations decreased obviously or were not detectable in most detected-samples at 6 d PI, without causing apparent symptoms. Perhaps, the results of this study indicate that the mice have an efficient protective immunity after oral challenge.

Salmonella cells have to attach to or form a close association with the intestinal epithelium in order to establish and persist in the gut and subsequently invade the underlying tissues[25,26]. *S. enteritidis* usually do not cause a disseminated systemic disease in humans, but clinically manifest as gastroenteritis and diarrhea[27]. The probability of establishment of persistent infection may involve a subtle interplay between host susceptibility and the challenge strain and does of *S. enteritidis*[10,28]. It has been reported that *S. enteritidis* can rapid distribution throughout the gastrointestinal tract, translocation to the mesenteric lymph nodes and spread to the liver and spleen of the mice. In the liver, the bacteria are ingested by the Kupffer cells, and then they invade the hepatocytes[29, 30]. In our studies, the spleens were positive at 12 h PI, but not the blood. It may be an indication that *S. enteritidis* cells were drained by the lymphatic system circulation (especially the Peyer's patchs in gut) priority. Also, recent studies have shown that some systemic spread of *salmonella* can occur without drainage through the lymphatic system and blood circulation[31]. Pathogen sampled subepithelial or even luminally by dendritic cells or CD18-expressing phagocytes can be transferred directly to the liver and spleen[32,33].

Previous studies showed that there is a association between *Salmonella* and intracellular survival in macrophages, which can be regarded as safe sites for bacterial multiplication[34,35]. However, how *S. enteritidis* survives within macrophages is unclear. It seems likely that the type Ⅲ secretion system encoded by *Salmonella* pathogenicity island 2 may play a major role[36,37]. One of the functions of *Salmonella* pathogenicity island 2 is to inhibit NADPH oxidase-dependent killing of *Salmonella*[38] . Also, it has been reported that the *S.* typhimurium genome encodes many mechanisms that allow resistance to the stressful environment encountered within the macrophage. Such stress include the production by NADPH oxidase of superoxide anion (O_2^-) and other reactive oxygen species (ROS), and by inducible nitric oxide (NO) synthase (iNOS) of NO and other reactive nitrogen species (RNS)[39,40]. A number of important bacterial pathogens infect, replicate, and persist within nucleated cells of the host, T-cell-mediated immunity has proven to be a critical factor in the effective clearance of many such intracellular bacterial pathogens. However, *Salmonella* can induce suppression of cellular responses[41]. Simultaneously, it has been

reported that chicken macrophages display differences in their responses to *S. enteritidis* and *S.* typhimurium, and contribute to the differential pathogenesis of these *Salmonella* serovars[42]. Also, it has been reported that *S. enteritidis* infection induce less inflammation resulting in a more commensal, while *S.* typhimurium infection can be cleared more rapidly by induction of inflammatory[43,44]. *S. enteritidis* resulted in increased splenic CD3 and reduced B populations, it was difficult to associate this increase with *S. enteritidis* clearance due to lack of any significant changes in $CD4^+$ of $CD8^+$ cells[45].The functions of the spleen and liver in filtration, immune responsiveness and activation of complement have been well documented. Spleen is a container for the lymphocyte-rich white pulp and macrophage-rich red pulp; it is comprised of distinctive B cells and macrophages. So, what we described above may be the reason for why significant number of *S. enteritidis* cells can persist over a long time in liver and spleen without causing apparent symptoms *in vivo* in this study. Up to day, the colonization mechanism of *S. enteritidis* in the gut and the internal organs are not clear and further studies should be carried out.

Interestingly, the gallbladder is a site of carriage in this study, it is also the storage site for bile. Our studies may be the first to report that *S. enteritidis* can persist for as long as 12 d PI in gallbladder in mice. Bile is produced in the liver and consists of many components, including bile salts, cholesterol, and bilirubin. Bile salts are detergents that aid in degradation and dispersion of lipids, and such make bile a good antimicrobial agent[7]. It has been reported that *Salmonella* is resistant to high concentrations of bile and individual bile salts[46]. From the liver, the *S.* typhimurium can be shed into the gallbladder, and this infection is frequently associated with gallbladder abnormalities, such as gallstones, also, this infection is often asymptomatic and can last for many years[5,6]. Biofilms have recently been implicated as the cause of many chronic infection in human[47]. *S. enteritidis* are capable of shielding themselves from environmental stress, host immune response and phagocytosis by the secretion of an apparently amorphous matrix of secreted polysaccharides[47-50]. This matrix provides a very stable environment and results in high levels of resistance to antimicrobial agents[46,50]. Often, it is difficult to clear the infection unless the substrate to which the bacteria are attached is removed[7]. In our studies, *S. enteritidis* cells can persist for 12 d PI with low concentrations, about 1.25×10^2 to 6.61×10^2 copies/0.2 mL. The gallbladder appeared to be gross lesion (such as swelling) at 20 h to 2 d PI. Interestingly, there were not any significant gross lesions over the 3-12 d PI period, although there was nearly the same number of *S. enteritidis* cells over the 12 d period.

FQ-PCR has become a potentially powerful alternative in microbiological diagnostics due to its simplicity, rapidity, reproducibility, and accuracy[51,52]. However, variation results may be due to either the PCR inhibitors, or a large amount of DNA from background organism DNA. In preliminary experiments, we used phenol/chloroform/isoamyl alcohol method to extract DNA of tissue from several control group samples (described above), and added 1.0×10^5 copies of the standard DNA for each. Finally, fluorimetic cycler measurements were performed as described above. The results showed that all the tests can obtain the expected data and the variability was statistically low, at < 4.3%. So, this methodology is very accuracy for studying on the distribution of *S. enteritidis* in the internal organs.

In conclusion, our results provide significant data for helping to clarify the pathogenic mechanism of *S. enteritidis* in the internal organs, and show that the liver and the spleen are the primary sites of invasion in normal mice after oral challenge, interestingly, the gallbladder is a site of carriage over a 12 d period in this study, to our knowledge, this is the first time reported, and future studies should be carried out.

COMMENTS

Background

There are over 2500 serovars in the genus *Salmonella*. It has been a public health concern for over 100 years, and the incidence of *Salmonella* infections has risen dramatically, especially those caused by *S. enteritidis*. Therefore, knowledge about *Salmonella* infection could be an additional means for decreasing the incidence of infection. Infection with *Salmonella* is usually started by oral ingestion of the pathogen, and is followed by bacterial colonization of the gut and invasion of internal tissues. As a matter of course, it is necessary to understand its mechanisms of action in the internal organs.

Research frontiers

To date, the regular distribution pattern for *S. enteritidis* in the internal organs of mice after oral challenge has not been established. FQ-PCR, as a rapid, sensitive technique for precise quantitation of nucleic acid, will be an ideal method to study the distribution of *S. enteritidis* in the internal organs.

Innovations and breakthroughs

These studies have identified the regular distribution pattern of *S. enteritidis* invasion in the internal organs, which is not described hitherto. Moreover, it may be the first time reported that the gallbladder is a site of carriage for *S. enteritidis* over a 12-d period.

Applications

This study will provide significant data for clarifying the pathogenic mechanisms of *S. enteritidis* in the internal organs, and may ultimately lead to new insights in prevention and therapy.

Peer review

The manuscript by Deng and colleagues represents a clear and straight-forward study into the distribution and persistence on *S. enteritidis* in the internal organs of infected mice. The hypothesis and methods are clearly presented, and the results will help future studies.

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RAPID COMMUNICATION

Factors associated with fasting plasma ghrelin levels in children and adolescents

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Abstract

AIM: To measure plasma ghrelin levels in children and adolescents, analyze the associated factors, and investigate the role of ghrelin in obesity, insulin resistance and reproductive physiology.

METHODS: A total of 283 subjects aged 4.8-15.8 year were enrolled. Fasting blood samples were collected and plasma ghrelin levels were measured by radioimmunoassay. Fasting glucose (FG), fasting insulin (FI), baseline testosterone (T), estradiol (E2), prolactin (PRL), luteinizing hormone (LH), follicle-stimulating hormone (FSH), serum total cholesterol (TC), triglyceride (TG), alanine aminotransferase (ALT) and uric acid (UA) were measured. Body mass index (BMI), insulin resistance by homeostasis model (HOMA-IR) and beta cell function by homeostasis model (HOMA-β) were calculated.

RESULTS: The median ghrelin level was 290 ng/L (15.0-1325.0 ng/L). Bivariate correlation analysis showed that ghrelin levels were inversely correlated with BMI, ALT, TG, UA, LH, FI and HOMA-IR (all $P < 0.05$). No other significant correlation was found between ghrelin levels and age, gender, TC, E2, FSH, PRL, FG and HOMA-β. Stepwise multiple regression analysis showed that only BMI and FI were independent determinants of plasma ghrelin levels in these children and adolescents ($P = 0.018$ and $P = 0.046$, respectively), which explained 25.4% of the variance.

CONCLUSION: These data suggest that the lower ghrelin levels in obese subjects may be the result of obesity and hyperinsulinemia, which is very common in obese subjects. Moreover, ghrelin may regulate human reproductive physiology indirectly.

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Key words: Ghrelin; Obesity; Body mass index; Insulin

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INTRODUCTION

Ghrelin, recently isolated from rat and human stomachs, is an endogenous ligand for growth hormone secretagogue receptor (GHSR) type 1a[1,2]. Recent studies have shown that ghrelin is secreted from hypothalamus, pituitary, endocrine pancreas islets, bowel, kidney, heart and testis[1,2]. Besides stimulating GH secretion, ghrelin has been reported to have other endocrine and non-endocrine actions, including stimulation of lactotroph and corticotroph secretion, effects on the gonadal axis, cardiovascular activity, influence on behavior and sleep, modulation of cell proliferation, repression of apoptosis, and orexigenic effects[3-7]. However, most of these studies used animal models and there were some discrepancies in the results. Thus, the regulation of ghrelin secretion and its specific role in endocrinology in humans, including the gonadal axis, insulin resistance, energy balance and obesity, are not completely understood.

In this study, we measured plasma ghrelin levels in Chinese children and adolescents, analyzed their associated factors, and investigated the role of ghrelin in obesity, insulin resistance and reproductive physiology.

MATERIALS AND METHODS

Materials

A total of 283 subjects aged 4.8-15.8 year (mean ± SD;

9.4 ± 2.4 year) were enrolled in this study. They included 133 males and 150 females. Body mass index (BMI) was 12.15-44.00 kg/m^2 (19.88 ± 6.23 kg/m^2) with a median BMI Z score of 0.52. Ninety-six individuals were prepubescent and 187 were pubescent, including 107 with Tanner Ⅱ stage breasts, 53 with Tanner Ⅲ, 19 with Tanner Ⅳ and eight with Tanner Ⅴ. Subjects with obesity were included and subjects with other endocrine diseases, viral hepatitis, kidney and infectious diseases were excluded. Written informed consent was obtained from the parents and the study was approved by the Ethics Committee of the Children's Hospital of Zhejiang University School of Medicine, China.

Plasma ghrelin measurement

Two-milliliter venous blood samples were obtained by venipuncture after an overnight fast and were placed in a tube with 30 μL 10% EDTA and 20 μL aprotinin. Plasma was isolated and stored at -70℃ until analysis. Plasma total ghrelin levels, including Ser 3-octanoyl and Ser 3-des-octanoyl ghrelin, were measured by radioimmunoassay. The commercially available kits were purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). The assay sensitivity and CV were 10 ng/L and 5%-10%, respectively.

Biochemistry markers

Fasting glucose (FG) and insulin (FI) levels were measured by glucose oxidase method and radioimmunoassay (Beijing North Biotechnology Invest, China). Insulin resistance by homeostasis model (HOMA-IR) and beta cell function by homeostasis model (HOMA-β) were calculated according to Matthews *et al*[8]: HOMA-IR = FG × FI/22.5 and HOMA-β = 20 × FI/(FG-3.5).

Baseline testosterone (T), estradiol (E2), prolactin (PRL), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured by chemiluminescence (Siemens, Gwynedd, UK). Serum total cholesterol (TC), triglyceride (TG), alanine aminotransferase (ALT) and uric acid (UA) were measured in the clinical laboratories of the Children's Hospital of Zhejiang University School of Medicine.

Body composition measurements

Body composition was measured in the morning after an overnight fast. Body weight was measured to the nearest 0.1 kg on an electronic scale, and body height was measured to the nearest 0.1 cm, with the subjects in underwear and barefoot. BMI was calculated as weight (kg) divided by height (m) squared.

Statistical analysis

Statistical analyses were conducted by using SPSS software (11.5 Version) (SPSS, Chicago, IL, USA). Quantitative data with normal distribution were presented as the mean ± SD, and data with skewness distribution were presented as the median (min-max). The T and E2 levels, which were lower than measured limits (both 20 ng/dL), were treated as the lower limit. The relation between fasting ghrelin levels and other factors was analyzed by bivariate correlation using the Pearson test. Stepwise multiple linear regression models were used to examine the determinants of plasma ghrelin levels. Differences were considered statistically significant at $P < 0.05$.

Table 1 Bivariate correlation analysis between fasting ghrelin levels (ng/L) and other factors

	r value	*P* value
Age (yr)	-0.107	0.073
Gender (male 1, female 0)	0.082	0.167
BMI Z score	-0.259	< 0.001
FI (mIU/L)	0.283	< 0.001
FG (mmol/L)	-0.108	0.085
HOMA-IR	0.266	< 0.001
HOMA-β	-0.049	0.447
ALT (U/L)	-0.192	0.002
TG (mmol/L)	-0.136	0.029
TC (mmol/L)	-0.051	0.258
UA (μmol/L)	-0.210	0.001
T (ng/L)	-0.149	0.016
E2 (ng/L)	-0.075	0.226
LH (IU/L)	-0.120	0.053
FSH (IU/L)	-0.063	0.281
PRL (μg/L)	-0.003	0.963

RESULTS

In 283 subjects, the levels of FG, FI, HOMA-IR and HOMA-β were 4.55 ± 0.64 mmol/L, 7.60 mIU/L (2.0-56.90 mIU/L), 1.57 (0.29-12.39) and 156.80 (-5240 to 10 640), respectively. The levels of ALT, TG, TC and UA were 18.0 U/L (6.0-369.0 U/L), 1.42 mmol/L (0.22-8.64 mmol/L), 4.18 mmol/L (2.12-7.81 mmol/L) and 297.75 ± 82.09 μmol/L, respectively. The levels of T, E2, LH, FSH and PRL were < 200.0 ng/L (< 200.0-2700 ng/L), < 20.0 ng/L (< 20-98.6 ng/L), 0.30 IU/L (0.10-98.60 IU/L), 2.40 IU/L (0.10-100.0 IU/L) and 13.30 μg/L (2.90-69.30 μg/L). The median ghrelin level was 290 ng/L (15.0-1325.0 ng/L).

Bivariate correlation analysis was performed between fasting ghrelin levels and other factors, including age, gender (female as 0, male as 1), BMI Z score, ALT, TG, TC, UA, T, E2, LH, FSH, PRL, FG, FI, HOMA-IR, and HOMA-β (Table 1). The results showed the ghrelin levels were inversely correlated with BMI, ALT, TG, UA, FI and HOMA-IR (all $P < 0.05$). LH, age and FG had a marginal relationship with ghrelin (P ranged from 0.053 to 0.085). No other significant correlation was found between ghrelin levels and gender, TC, E2, FSH, PRL and HOMA-β.

Stepwise multiple regression analysis included all the factors which showed $P < 0.1$ in the bivariate correlation analysis. We found that only FI and BMI were independent determinants of plasma ghrelin levels in these children and adolescents, which explained 22.7% of the variance (Table 2). Plasma ghrelin levels were inversely associated with FI and BMI Z score ($P = 0.026$ and $P = 0.048$, respectively). HOMA-IR, T, LH, ALT, TG, UA and age were excluded in the equations (all $P > 0.05$).

Table 2 Multiple linear regression analysis for determinant of plasma ghrelin levels

Variable	Coefficient (Beta)	*r* value	*P* value
FI (mIU/L)	-1.208	2.244	0.026
BMI Z score	-1.107	1.985	0.048
FG (mmol/L)	-0.203	1.857	0.065
ALT (U/L)	-0.099	1.252	0.212
T (ng/L)	-0.081	1.125	0.262
HOMA-IR	0.099	1.251	0.277
UA (μmol/L)	-0.046	0.574	0.567
Age (yr)	0.026	0.371	0.711
LH (IU/L)	-0.017	0.237	0.813
TG (mmol/L)	0.002	0.029	0.977

r^2 for the model = 0.227.

DISCUSSION

Ghrelin has recently been identified as a ligand of GHSR type 1a. It has been reported to have endocrine and nonendocrine actions[3-7], although there have been some contrary results. Also, the regulation of ghrelin secretion and its exact role have not yet been fully elucidated. In this study, we measured the plasma ghrelin levels in 283 Chinese children and adolescents and investigated the associated factors. We found that the median ghrelin level was 290 ng/L (15.0-1325.0 ng/L), which was similar with that reported previously[9,10].

We also found that BMI Z scores were inversely correlated with fasting plasma ghrelin levels, both by bivariate correlation and multiple regression analyses. This confirmed the view that ghrelin is closely associated with obesity. Ghrelin has been considered as a cause of obesity in some studies, on account of the fact that ghrelin has orexigenic effects in both rats and humans[11,12]. However, the results of most studies in humans, similar to our own, have demonstrated that ghrelin levels are negatively correlated with BMI Z score[9,13]. Moreover, Marzullo *et al* have reported that active ghrelin levels in subjects with obesity are lower than those in lean subjects[14]. These findings are contrary to the view that ghrelin is a factor in obesity. We speculate that the lower ghrelin levels in obesity are part of negative feedback to inhibit appetite and body weight, but not the primary cause of obesity[15]. This is also supported by the fact that circulating ghrelin levels increase in anorexia and cachexia[16-18]. However, animals without ghrelin do not have significantly altered body weight or food intake when compared with their wild-type littermates[19,20]. This suggests that it is a part of a reversible feedback mechanism, but not a determinant factor. Further studies are required to investigate the effect and the mechanism of ghrelin deficiency in individuals with obesity.

We also report that FI, HOMA-IR, ALT, UA and TG (but not glucose or HOMA-β) were negatively associated with plasma ghrelin levels. Moreover, FI was an independent factor of ghrelin levels by multiple regression analysis. These findings are supported by several previous studies[21,22] and suggest that insulin suppresses circulating ghrelin, independently of glucose. Hence, we speculate that hyperinsulinemia, which is very common in children and adolescents with obesity[23-25], might partly explain their lower ghrelin levels.

Some previous studies have suggested that ghrelin plays a role in the regulation of reproductive physiology. Expression of ghrelin in rat and human testes has been investigated[26,27], and some studies have reported that ghrelin levels are related to GnRH and androgens[28-31]. However, some animal studies have shown that ghrelin stimulates GnRH and/or androgens *in vitro*[28,29], but inhibits these hormones *in vivo*[30,31]. However, similar studies investigating the relationship between ghrelin and the gonadal axis in humans are rare, and the actual role of ghrelin in reproductive physiology is still unclear. In this study, we found that T and LH (but not E2 and FSH) were negative associated with fasting plasma ghrelin levels, although T and LH were not independent factors for ghrelin levels in stepwise regression analysis. This finding suggests that ghrelin regulates reproductive physiology in humans indirectly.

There are several limitations in this current study. Firstly, the sample may have been too small to find major differences. Secondly, we measured total circulating ghrelin, including Ser 3-octanoyl and Ser 3-des-octanoyl ghrelin, but not the active form of ghrelin. Thirdly, the actual correlation coefficients between serum ghrelin levels and BMI Z scores and FI were low. These imply that some other factors play a role in regulating circulating ghrelin levels.

In summary, our data suggest the lower ghrelin levels in subjects with obesity are the result of obesity and hyperinsulinemia, which is very common in these subjects. Moreover, ghrelin may regulate human reproductive physiology indirectly. Further studies are required to improve our understanding of the ghrelin effect and regulation of ghrelin secretion.

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COMMENTS

Background

Ghrelin is an endogenous ligand for growth hormone secretagogue receptor type 1a and has some endocrine and non-endocrine actions. However, the regulation of ghrelin secretion and its specific role in endocrinology in humans, including the gonadal axis, insulin resistance, energy balance and obesity, are not completely understood.

Research frontiers

Worldwide, obesity has reached epidemic proportions. Obesity also increases the risk of developing a number of chronic diseases including insulin resistance, type 2 diabetes, hypertension, hypercholesterolemia, stroke, and heart attack. Hence, analysis of the relationship between ghrelin levels, obesity and insulin resistance is necessary.

Related publications

The prevalence of obesity has increased in China, which is closely associated with

metabolic syndrome (*Int J Obes* 2007; 31: 15-22). Some studies have reported the relationship between obesity, adipocytokines and cytokines (*Endocrin J* 2005; 52: 519-524. *Indian Pediatr* 2007; 44: 275-279).

Innovations and breakthroughs

The lower ghrelin levels in subjects with obesity may be the result of obesity and hyperinsulinemia, which is very common in these subjects. Moreover, ghrelin might regulate human reproductive physiology indirectly.

Applications

Regulating ghrelin levels may be beneficial for improving insulin sensitivity, controlling body weight, or even reproductivity.

Terminology

Insulin resistance is a condition in which the cells of the body become resistant to the effects of insulin, that is, the normal response to a given amount of insulin is reduced. As a result, higher levels of insulin are needed in order for insulin to have an effect. Almost all individuals with type 2 diabetes mellitus and many with hypertension, cardiovascular disease, and obesity are insulin resistant.

Peer review

This study is of particular importance to childhood health and obesity, and correlates many factors with plasma ghrelin in the fasting state. It suggests an interesting hypothesis linking hyperinsulinemia and obesity to lower ghrelin levels.

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RAPID COMMUNICATION

Portalsystemic hemodynamic changes in chronic severe hepatitis B: An ultrasonographic study

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Abstract

AIM: To evaluate portalsystemic hemodynamic changes in chronic severe hepatitis B.

METHODS: Hemodynamic parameters included portal vein diameter (PVD), portal vein peak velocity (PVPV), portal vein volume (PVV), spleen length (SPL), spleen vein diameter (SPVD), spleen vein volume (SPVV) and umbilical vein recanalization. They were measured by Color Doppler ultrasonography in 36 patients with chronic severe hepatitis B, compared with 51 normal controls, 61 patients with chronic hepatitis B, 46 patients with compensable cirrhosis, and 36 patients with decompensable cirrhosis.

RESULTS: In the group of chronic severe hepatitis B, PVD (12.38 ± 1.23 mm) was significantly different from the normal control, compensable cirrhosis and decompensable cirrhosis groups (P = 0.000-0.026), but not significantly different from the chronic hepatitis group. PVPV (16.15 ± 3.82 cm/s) dropped more significantly in the chronic severe hepatitis B group than the normal control, chronic hepatitis B and compensable cirrhosis groups (P = 0.000-0.011). PVV (667.53 ± 192.83 mL/min) dropped significantly as compared with the four comparison groups (P = 0.000-0.004). SPL (120.42 ± 18.36 mm) and SPVD (7.52 ± 1.52 mm) were longer in the normal control and chronic hepatitis B groups (P = 0.000-0.009), yet they were significantly shorter than those in the decompensable cirrhosis group (P = 0.000). SPVV (242.51 ± 137.70 mL/min) was also lower than the decompensable cirrhosis group (P = 0.000). The umbilical vein recanalization rate (75%) was higher than the chronic hepatitis B and compensable cirrhosis groups. In the course of progression from chronic hepatitis to decompensable cirrhosis, PVD, SPL and SPVD gradually increased and showed significant differences between every two groups (P = 0.000-0.002).

CONCLUSION: Patients with chronic severe hepatitis B have a tendency to develop acute portal hypertension, resulting in significantly reduced portal vein perfusion. Observation of the portalsystemic hemodynamic changes may be contributed to the disease progression of chronic liver disease.

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Key words: Color doppler ultrasonography; Portalsystemic hemodynamics; Chronic severe hepatitis B

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INTRODUCTION

Chronic severe hepatitis carries a very high mortality rate (60%-80%), and causes about 22 600 deaths in China annually. It is widely known that progression of liver diseases is accompanied by histological changes of the hepatic parenchyma, such as inflammation, necrosis and fibrosis, as well as hemodynamic changes. Because hepatic circulation closely correlates to chronic liver disease, confirmation of the changes in the hepatic circulation is important for clarifying the disease progression. Liver biopsy is the gold standard for the grading and staging of the viral hepatitis. However, this procedure causes discomfort, and is invasive for the patients, especially those with prolonged prothrombin time[1-3]. Ultrasonography has the advantages of low cost, easy operation and high acceptability by the patients. It can provide not only valuable information on the morphological changes of the liver but also liver hemodynamics by color Doppler flow imaging[4-7]. The introduction of color Doppler

ultrasonography has been an important breakthrough in the noninvasive evaluation of splanchnic hemodynamics. Both qualitative and quantitative evaluations of the portalsystemic circulation are being widely used in patients with chronic hepatitis and cirrhosis[8-10]. However, until the present, there have been only a few reports on portalsystemic hemodynamic changes in chronic severe hepatitis B in the literature. Furthermore, there is no consensus on the role of Doppler parameters in grading the severity of chronic liver disease[11-16]. The purpose of this study is to evaluate portalsystemic hemodynamic changes in chronic severe hepatitis B non-invasively, and to delineate more definitively the role of Doppler parameters in patients with chronic hepatitis B and cirrhosis, as compared with normal subjects, chronic hepatitis B, compensable cirrhosis and decompensable cirrhosis patients.

MATERIALS AND METHODS

Subjects and diagnostic criteria

Between April 2005 and August 2007, 230 subjects were recruited in this study. Among them, 191 were males and 39 were females, with an average age of 34.7 ± 13.5 years (ranging from 10 to 77 years). According to the program of prevention and cure for viral hepatitis amended by the Chinese Society of Infectious Disease and Parasitology and the Chinese Society of Hepatology of Chinese Medical Association in the Xian meeting held in September 2000[17], the subjects were divided into five groups: 36 patients with chronic severe hepatitis; 51 normal controls; 61 patients with chronic hepatitis B; 46 patients with compensable cirrhosis; and 36 patients with decompensable cirrhosis. There were no significant differences in the gender and age among these five groups. Diagnoses of patients in the control groups except the normal group were made on the basis of pathological examination of liver biopsy specimens and laboratory findings. Ethical approvals of the liver biopsy were obtained before the operations. Subjects in the normal control group had normal hepatic functions, and no histories of chronic liver disease. Compensable cirrhosis belonged to Child-Pugh A, the diagnostic criteria included A $\geqslant$ 35 g/L, Bil <35 μmol/L, PTA > 60%, and portal hypertension but no ascites, hepatic encephalopathy and upper gastrointestinal bleeding. Decompensable cirrhosis belonged to Child-Pugh B, C, the diagnostic criteria included serious hepatic function abnormalities, such as A < 35 g/L, A/G < 1.0, Bil > 35 μmol/L, PTA < 60%, and ascites, hepatic encephalopathy, upper gastrointestinal varices or bleeding. Diagnostic criteria of chronic severe hepatitis B[18] included the basic conditions, i.e. the course of hepatitis B surface antigen-positive exceeded six months and the level of serum bilirubin in liver failure index exceeded ten times compared by normal level (17.1 μmol/L), which was above 171 μmol/L. Two additional conditions included (at least one liver failure index or more than one indexes below): (1) Prothrombin activity < 40%; (2) Hepatic encephalopathy; (3) Ascites; (4) Progressive reduction in liver size; (5) Hepatorenal syndrome. Exclusion criteria for patients in this study were autoimmunity hepatitis, alcoholic hepatitis, and the abnormality of cardiac and renal function accompanied by systemic hemodynamic changes.

Doppler measurements

All subjects were tested in the morning after an overnight fast and informed consents were obtained. All Doppler studies were performed with a color and pulsed Doppler unit (BIOSOUND AU4) armed with a 3.5-5.0 MHz convex probe. According to our previous study with the aim to select valuable ultrasonographic predictors for the evaluation of hepatic inflammation and fibrosis degree in chronic hepatitis[10], we selected the following Doppler parameters for this study: portal vein diameter (PVD), portal vein peak velocity (PVPV), portal vein volume (PVV), spleen length (SPL), spleen vein diameter (SPVD), spleen vein volume (SPVV), and umbilical vein recanalization.

The conditions of Doppler measurement: the sampling size was nearly equal to the vessel diameter, the angle between the Doppler beam and the longitudinal axis of the vessel was kept at less than 60°. Portalsystemic Doppler parameters were automatically determined for all samples of the Doppler signal that lasted for more than two cardiac cycles. Doppler ultrasound parameters were measured in triplicate, and the results were averaged.

In order to reduce inter-observer variability to a non-significant level, all examinations were performed by one of the author's that was blind to the patients' clinical details. Feasibility of a satisfactory Doppler US examination was required for each patient recruited in this study. All patients were not affected by cardiopulmonary, circulatory, renal, or abdominal diseases and were not taking medications. The formula of blood volume was $(V) = 1/4\pi \times a \times b \times 0.57\ V_{peak} \times 60$ (mL/min) a: antero-posterior diameter of vessel, b: transverse diameter of vessel, Unit: cm, V_{peak}: peak velocity.

Statistical analysis

Results were described as mean ± SD and analyzed on the computer with SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). Differences in means among the groups were evaluated by one-way ANOVA and LSD. Proportions were evaluated by χ^2-test. P values < 0.05 were considered statistically significant.

RESULTS

Means of the portalsystemic hemodynamic parameters in different groups are shown in Table 1.

Differences of the portalsystemic hemodynamic parameters in different groups are shown in Table 2.

PVD in the chronic severe hepatitis group was significantly different from the normal control, compensable cirrhosis and decompensable cirrhosis groups, but not significantly different from chronic hepatitis B group. Among the four comparison groups, PVD gradually increased and was significantly different between every two groups with disease progression; PVPV in the chronic severe hepatitis group evidently decreased in comparison to the normal control, chronic hepatitis B

Table 1 Means of portalsystemic hemodynamic parameters in different groups

Group	*n*	PVD (mm)	PVPV (cm/s)	PVV (mL/min)	SPL (mm)	SPVD (mm)	SPVV (mL/min)
NCG	51	11.13 ± 0.91	26.15 ± 4.45	878.52 ± 177.07	94.88 ± 9.91	5.65 ± 0.96	160.66 ± 65.87
CHG	61	12.09 ± 1.14	22.42 ± 5.17	926.51 ± 188.24	105.64 ± 14.29	6.50 ± 1.44	250.96 ± 182.35
RCG	46	13.05 ± 0.87	18.52 ± 2.64	845.21 ± 173.67	120.28 ± 17.88	7.62 ± 1.84	309.43 ± 195.20
ICG	36	14.25 ± 2.42	16.95 ± 3.52	960.89 ± 472.91	152.89 ± 35.80	10.23 ± 3.06	625.43 ± 474.59
SCHG	36	12.38 ± 1.23	16.15 ± 3.82	667.53 ± 192.83	120.42 ± 18.36	7.52 ± 1.52	242.51 ± 137.70

NCG: Normal control group; CHG: Chronic hepatitis B group; RCG: Compensable cirrhosis group; ICG: Decompensable cirrhosis group; SCHG: Chronic severe hepatitis B group; PVD: Portal vein diameter; PVPV: Portal vein peak velocity; PVV: Portal vein volume; SPL: Spleen length; SPVD: Spleen vein diameter; SPVV: Spleen vein volume.

Table 2 Differences of portalsystemic hemodynamic parameters in different groups

Group and group	PVD (*P*)	PVPV (*P*)	PVV (*P*)	SPL (*P*)	SPVD (*P*)	SPVV (*P*)
NCG and CHG	0.000	0.000	0.424	0.033	0.014	0.086
NCG and RCG	0.000	0.000	0.576	0.000	0.000	0.008
NCG and ICG	0.000	0.000	0.174	0.000	0.000	0.000
NCG and SCHG	0.000	0.000	0.001	0.000	0.000	0.177
CHG and RCG	0.000	0.000	0.184	0.000	0.002	0.252
CHG and ICG	0.000	0.000	0.579	0.000	0.000	0.000
CHG and SCHG	0.320	0.000	0.000	0.001	0.009	0.881
RCG and ICG	0.000	0.090	0.061	0.000	0.000	0.000
RCG and SCHG	0.026	0.011	0.004	0.977	0.803	0.259
ICG and SCHG	0.000	0.413	0.000	0.000	0.000	0.000

NCG: Normal control group; CHG: Chronic hepatitis B group; RCG: Compensable cirrhosis group; ICG: Decompensable cirrhosis group; SCHG: Chronic severe hepatitis B group; PVD: Portal vein diameter; PVPV: Portal vein peak velocity; PVV: Portal vein volume; SPL: Spleen length, SPVD: Spleen vein diameter; SPVV: Spleen vein volume.

and compensable cirrhosis groups, but not significantly different from the decompensable cirrhosis group. Except the compensable and decompensable groups, PVPV gradually decreased and was significantly different between every two groups with disease progression; PVV in the chronic severe hepatitis group significantly decreased, but the decrease did not reach significant difference among the four control groups; SPL in the chronic severe hepatitis group remarkably increased in comparison to the normal control and chronic hepatitis B groups but remarkably decreased in comparison to the decompensable cirrhosis group, and there was no significant difference as compared with the compensable cirrhosis group. SPL gradually increased and was significantly different between every two groups with disease progression; SPVD in the chronic severe hepatitis group evidently increased in comparison to the normal control and chronic hepatitis B group, but evidently decreased in comparison to the decompensable cirrhosis group and there were no significant difference as compared with the compensable cirrhosis group. SPVD gradually increased and was significantly different between every two groups with disease progression; SPVV in the chronic severe hepatitis group significantly decreased in comparison to the decompensable cirrhosis group. There was no significant difference in the normal control, chronic hepatitis B and compensable cirrhosis groups.

The umbilical vein recanalization rates in different groups are shown in Table 3.

Table 3 Umbilical vein recanalization rates in different groups

Group	Umbilical vein recanalization rate
NCG	0% (0/51)
CHG	0% (0/61)
RCG	22% (10/46)
ICG	56% (20/36)
SCHG	75% (27/36)

NCG: Normal control group; CHG: Chronic hepatitis B group; RCG: Compensable cirrhosis group; ICG: Decompensable cirrhosis group; SCHG: Chronic severe hepatitis B group.

Table 4 Differences of umbilical vein recanalization rates in different groups

Group and group	χ^2 values	*P*
RCG and ICG	9.954	0.002
RCG and SCHG	23.135	0.000
ICG and SCHG	3.003	0.083

NCG: Normal control group; CHG: Chronic hepatitis B group; RCG: Compensable cirrhosis group; ICG: Decompensable cirrhosis group; SCHG: Chronic severe hepatitis B group.

Differences of the umbilical vein recanalization rates in different groups are shown in Table 4.

The umbilical vein recanalization rate in the chronic severe hepatitis group significantly increased in comparison to the compensable cirrhosis group and there was no significant difference from decompensable cirrhosis group. There were no umbilical vein recanalizations in the normal control and chronic hepatitis B groups.

DISCUSSION

Chronic severe hepatitis B refers to patients with evidence of chronic liver disease that then develops acute decompensation of liver function. Usually, the acute decompensation of liver function is caused by one or more potentially reversible precipitating events, such as superimposed acute hepatic necrosis due to other hepatotropic virus infections drugs, alcohol, or ischemia sepsis, worsening renal function, and gastrointestinal bleeding. Chronic severe hepatitis often includes both acute-on-chronic liver failure and chronic liver failure[19]. In China,

the majority of fulminant hepatitis is chronic liver failure caused by hepatitis virus B. Because of the underlying pathological changes of chronic hepatitis or liver cirrhosis, when liver failure leads to massive necrosis of the liver, treatments become more difficult[20]. Obviously, chronic severe hepatitis B threatens the health of hepatitis patients because of its poor prognosis and high mortality.

Our results showed that there were no significant differences in PVV among the four control groups (P = 0.178-0.580), but PVV in the chronic severe hepatitis B group obviously decreased and was significantly different from the four comparison groups (P = 0.000-0.004). This reason may be, in our opinion, predominantly due to mechanical factors. As the liver disease progresses from chronic hepatitis to decompensable cirrhosis, hepatocellular inflammation, necrosis, development of regenerative nodules and collagen deposition, lobule reconstruction occur. These pathological changes may be resulted in gradual increase of portal vein circumfluence resistance, gradual decrease of portal vein velocity, and gradual dilation of portal vein diameter. In this process, dilation of the portal vein diameter may counteract the decrease in portal velocity, thus PVV was able to retain stability in a certain extent, so that hepatic perfusion could be maintained. However, in chronic severe hepatitis B, because of patchy areas of confluent massive hepatocellular necrosis, condensation of the reticulin framework of the lobule occurs in a short time, hepatic sinus interval becomes narrow, and even obstructed, and blood vessel bed decreases. Rapid increase of portal vein circumfluence resistance may be the result of these pathological changes, and may result in an obvious decrease in PVPV and acute portal hypertension formation. It was reported that patients with acute hepatitis and fulminant hepatitis developed portal hypertension with the aggravation of hepatic inflammation degrees[21,22]. Additionally, PVD was difficult to dilate to compensate for PVPV decrease in time, so PVV significantly dropped in comparison to the control groups. Because PVV in chronic severe hepatitis significantly decreased (only 76% of the normal group), hepatic perfusion derived from the portal vein was insufficient, and hepatocellular ischemia and hypoxia deteriorated tissue injury. Meanwhile, chronic severe hepatitis B could occur as a result of hepatic ischemia and hypoxia, and finally formed a vicious circle severely affecting the prognosis. This hypothesis may provide a theoretical base for clinical experience of dilating hepatic vessel, relieving portal vein resistance, increasing PVV and perfusion, and improving hepatic microcirculation[23].

There was no significant difference in PVPV between the chronic severe hepatitis B and decompensable cirrhosis groups (P = 0.413). The reason might be that PVPV in the two groups partly overlapped. It was well known that chronic severe hepatitis B included patients who had decompensable cirrhosis with < 40% prothrombin activity and total bilirubin > 171 μmol/L.

In the progression from chronic hepatitis to decompensable cirrhosis, PVD, SPL and SPVD gradually increased and showed significant difference between every two groups (P = 0.000-0.002). Our results revealed that PVD, SPL and SPVD were useful predictors of the disease progression from chronic hepatitis to decompensable cirrhosis. This was identical with O'Donohue[24].

Normally, after interruption of the placental circulation at birth, the umbilical vein collapses and forms the ligamentum teres in the adult. However, when portal hypertension occurs, the umbilical vein may recanalize and serve as a collateral route[25,26]. It was recognized in 10%-29% of patients with portal hypertension[27]. In our results, in the progression from chronic hepatitis to decompensable cirrhosis, the rate of umbilical vein recanalization gradually increased, but it was 75% (27/36) in the chronic severe hepatitis B group, which was obviously higher than that in the control groups. We considered that from chronic hepatitis to decompensable cirrhosis, portal pressure slowly rose and chronic portal hypertension formed, extensive collateral channels opening, portal vein dilation and spleen enlargement occurred. These factors could decompress portal system. However, in chronic severe hepatitis B, PVD and SPL could not compensate in time due to acute portal hypertension formation. Decompression of the portal system had to rely on collateral circulation formation such as umbilical vein recanalization. This was likely to be the reason for the higher rate of umbilical vein recanalization in chronic severe hepatitis.

In addition, there were other factors responsible for portalsystemic hemodynamic changes. Liver circulation included portal vein and hepatic artery perfusion[28,29]. It was the limitation in this study that portal vein perfusion was measured alone. In recent years, some new techniques and methods have been introduced to the hemodynamic study in liver. For example, hepatic vein transit time was measured by spectral Doppler or pulse-inversion imaging[30,31].

In conclusion, patients with chronic severe hepatitis B have a tendency to develop acute portal hypertension, resulting in an obvious decrease in portal vein perfusion. Observation of portalsystemic hemodynamic changes may contribute to the disease progression of chronic liver disease.

COMMENTS

Background

The introduction of color Doppler ultrasonography has been an important breakthrough in the noninvasive evaluation of splanchnic hemodynamics. The purpose of this study is to evaluate portalsystemic hemodynamic changes in chronic severe hepatitis B non-invasively, as compared with normal subjects, chronic hepatitis B, compensable cirrhosis and decompensable cirrhosis patients.

Research frontiers

Both qualitative and quantitative evaluations of the portalsystemic circulation are being widely used in patients with chronic hepatitis and cirrhosis. However, up to the present, there have been only a few reports on portalsystemic hemodynamic changes in chronic severe hepatitis B in the literature. Furthermore, there is no consensus on the role of Doppler parameters in grading the severity of chronic liver disease.

Innovations and breakthroughs

The article found that portal vein perfusion obviously decreased in chronic severe hepatitis B; portal vein diameter (PVD), spleen length (SPL), spleen vein diameter (SPVD) gradually increased and showed significant difference between every two groups in the progression from chronic hepatitis to decompensable cirrhosis.

Applications

This study was of important clinical values of evaluation portalsystemic hemodynamic changes in chronic severe hepatitis B and proved that Doppler

parameters could play an important role in grading the severity of chronic liver disease.

Terminology

Chronic severe hepatitis B refers to patients with evidence of chronic liver disease that then develops acute decompensation of liver function.

Peer review

This study was of important clinical values, which could comprehensively clarify portalsystemic hemodynamic changes in chronic severe hepatitis B, and grade the severity of chronic liver disease from chronic hepatitis to decompensable cirrhosis.

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CASE REPORT

Perivascular epithelioid cell tumor of the liver coexisting with a gastrointestinal stromal tumor

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Abstract

Approximately 10% of patients with gastrointestinal stromal tumors (GIST) develop other neoplasms, either synchronously or metachronously. In this report we describe coexistence of a gastrointestinal stromal tumor and a hepatic perivascular epithelioid cell tumor (PEComa) in a 51-year-old woman with no evidence of tuberous sclerosis. A subcapsular hepatic nodule (0.8 cm in diameter) was found during surgery for symptomatic gastric neoplasm (15 cm in diameter) arising from the lesser curvature. Both tumors revealed histomorphological and immunohistochemical features confirming a diagnosis of a small incidental hepatic PEComa and a high risky extramural gastric GIST, respectively. The patient remained disease-free 25 mo after surgery with no evidence of tumor recurrence or new neoplasms. To our knowledge, this is the first report of PEComa in a patient with GIST. Hepatic lesions detected synchronously or metachronously in patients with GISTs may represent histogenetically distinct lesions and should be sampled to confirm or exclude metastatic GISTs.

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Key words: Gastrointestinal stromal tumors; PEComa; Coexistence; Liver; Synchronous

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) represent the most common mesenchymal neoplasms of the gastrointestinal (GI) tract with an estimated incidence of 14.5 cases/million inhabitants[1]. Histologically, GISTs may show pure spindle cells, epithelioid, mixed and rarely pleomorphic morphology and are characteristically strongly immunoreactive for c-kit (CD117). GISTs occur more frequently in the stomach (60%-70%), small intestine (20%-30%), colorectum and esophagus (together ≤ 10%). Metastases predominantly occur in the peritoneum and liver[2].

Perivascular epithelioid cell tumors (PEComas) belong to a family of mesenchymal neoplasms including angiomyolipoma, clear-cell "sugar" tumors of the lung and other organs, lymphangiomyomatosis (LAM) and a rare group of morphologically and immunophenotypically similar lesions that may affect soft tissues, viscera and bones[3-5]. The term PEC (perivascular epithelioid cell) was coined by Bonetti *et al*[6] to describe the epithelioid cells found in these neoplasms. Primary hepatic PEComas are extremely rare[7], the majority of them were reported as monotypic or epithelioid angiomyolipoma.

Synchronous or metachronous malignant neoplasms are detected in almost 10% of GIST patients. In this regard, the majority of GISTs associated with other neoplasms are incidentally detected during surgery for intra-abdominal cancers. Hepatic neoplasm during GIST surgery is exceptionally rare. In this report, we describe the first case of GIST with a synchronously diagnosed hepatic PEComa, both of them were completely surgically resected.

CASE REPORT

A 51-year-old woman sought medical care in November 2004 due to pain in the left hypochondrium for 2 mo. She reported a weight loss of 7 kg during that period. Physical examination revealed no signs of the tuberous sclerosis complex (TSC). A computerized tomography (CT) of the

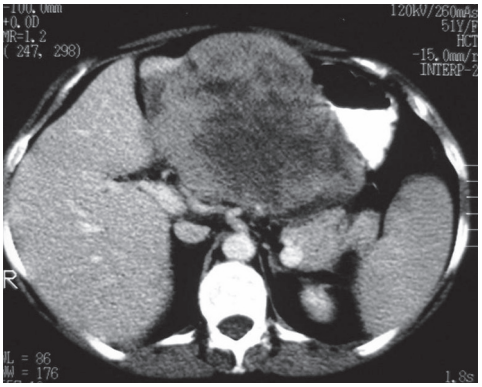


Figure 1 Computed tomography of the abdomen showing a 15-cm heterogeneous, solid expansile, regularly-shaped lesion located in the epigastrium and in contact with the left hepatic lobe and gastric wall.

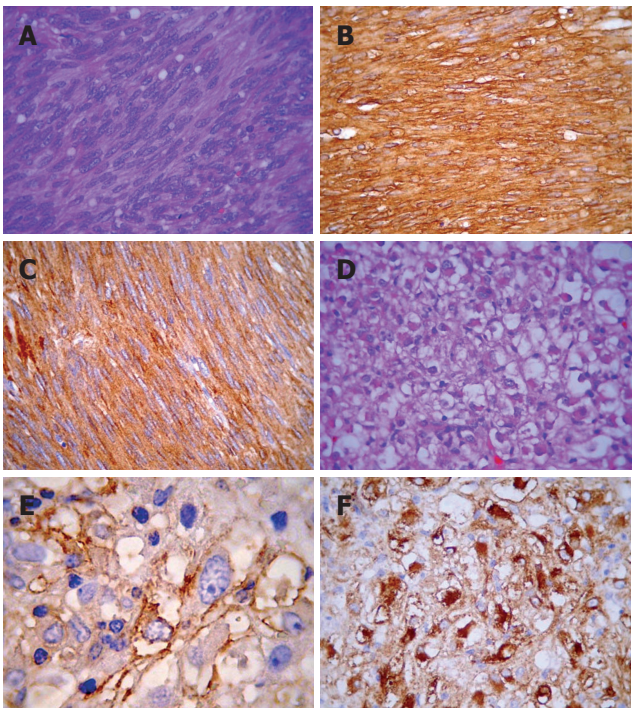


Figure 2 Morphological and immunohistochemical features of gastric GIST (**A-C**) and hepatic PEComa (**D-F**). **A**: GIST with characteristic spindled bipolar cells with occasional paranuclear cytoplasmic vacuoles; **B** and **C**: Strong staining for CD117 and CD34, respectively; **D**: Hepatic PEComa with a monotonous epithelioid morphology with cytoplasmic clearing that is hardly distinguishable from epithelioid GIST (note scattered cells with rhabdoid cytoplasm); **E**: A coarsely granular HMB45 reactivity; **F**: HH35 showing a strong paranuclear cytoplasmic reactivity.

abdomen showed a 15 cm well circumscribed solid expansile epigastric mass in contact with the left hepatic lobe and the gastric wall (Figure 1). CT of the thorax and pelvis did not show metastasis. At surgery, the mass was found to be attached to and infiltrating the gastric wall near the small curvature and could be successfully resected together with the large omentum and adjacent gastric wall involving the tumor. During surgery, a small subcapsular nodule located in the left hepatic lobe near the large gastric tumor was detected and resected. No tumor was found in the kidneys. The patient had a total abdominal hysterectomy for uterine leiomyomatosis in December 2006. At present, 25 mo after the surgery, she is under regular clinical follow-up with no evidence of tumor recurrence or metastasis.

Table 1 Summary of immunohistochemical features of the two tumors surgically excised

Antigen (Antibody clone)[1]	Dilution	PEComa	GIST
CD117	1:600	-	+
CD34	1:50	-	+
Vimentin	1:350	+	+
Cytokeratins (AE1/AE3)	1:250	-	-
HHF-35	1:2000	+	-
HMB-45	1:40	+	-
S100 protein	1:200	-	+

[1]DakoCytomation, Carpinteria, California, USA.

The resected gastric tumor measured 15.5 cm × 13.0 cm × 11.5 cm and was highly cellular, consisting of fascicles of spindled cells with elongated nuclei and finely granulated chromatin. Mitotic activity was brisk (3 mitoses/10 high-power fields). Cellular atypia was minimal and necrosis was absent (Figure 2A). Immunohistochemistry showed a strong expression of CD117 and CD34 (Figure 2B and C). The hepatic nodule measured 0.8 cm × 0.6 cm and was located near the hepatic surface, partly covered by its capsule. Histology showed sheets of large epithelioid cells with abundant eosinophilic, sometimes clear cytoplasm and mildly pleomorphic nuclei with evident nucleoli. A prominent feature was the presence of numerous dilated vascular spaces with a hemangiopericytic pattern. Necrosis or mitotic figures were not observed (Figure 2D). Immunohistochemistry showed a strong expression of HMB-45 and muscle specific actin (HHF-35), establishing a diagnosis of hepatic PEComa (Figure 2E and F). Table 1 summarizes the immunohistochemical data for both lesions.

DISCUSSION

The diagnosis of synchronous or metachronous neoplasias is not uncommon in clinical practice. Genetic predisposition and exposure to specific etiological or risk factors may be responsible for the association of histogenetically distinct tumors[8]. In such a circumstance, a second neoplasm developing in a patient with a known history of cancer is at a special risk of being misinterpreted as a metastatic disease, in particular, if this metachronous neoplasm presented at a site typically involving metastasis from the previously treated cancer, underscoring the need for sampling and a more careful interpretation of clinical, radiological and histopathological findings.

It was reported that the occurrence of synchronous and metachronous neoplasms is approximately 10% in patients diagnosed with GISTs and half of the cases can be diagnosed synchronously[9]. Gastrointestinal tract carcinomas are most frequently associated with GIST[9,10].

The presence of hepatic nodules in patients with a GIST must be carefully evaluated using appropriate immunohistochemical marker panel in order to distinguish metastatic GIST from other histogenetically distinct lesions (PEComa in the current case). Synchronous or metachronous primary hepatic neoplasms found incidentally in GIST patients are exceptionally rare with ⩽ 5 cases reported so far, including four hepatocellular

carcinomas and one inflammatory pseudotumor[9,11,12].

GISTs may enter the differential diagnosis of PEComas, since both can affect intra-abdominal structures. Due to their heterogeneous morphologic spectrum, GIST and PEComa may show largely overlapping features, thus representing a great differential diagnostic challenge for histopathologists. PEComas can be erroneously mistaken for GIST due to the monotonous epithelioid morphology and cytoplasmic clearing seen in a subset of both, combined with positive CD117 observed in some PEComas[5,7,13] and lacking or weak CD117 expression observed in a subset of epithelioid GISTs harboring mutations in platelet-derived growth factor receptor A (*PDGFRA*)[14]. Evert *et al*[13] reported a case of a malignant PEComa of the rectovaginal space mimicking a GIST and advocated the use of melanocytic markers in cases of suspected GIST with less than 50% of tumor cells expressing CD117. However, the characteristic morphology of PEComas may facilitate diagnosis in most of the cases. In difficult cases, immunohistochemistry would confirm the diagnosis of PEComa, as PEComas commonly show a characteristic myomelanocytic marker profile, occasionally with a characteristic perivascular distribution of HMB45 positive cells. PEComas commonly consist of epithelioid to elongated cells with abundant clear to granular eosinophilic cytoplasm with a perivascular organization of PECs in some of the cases[15]. While expression of HMB45 is a regular feature in most PEComas, smooth muscle markers and S100 show a rather heterogeneous and inconsistent reactivity pattern. CD117 expression in PEComas shows variable results, however, this finding is probably rare (5%) when appropriate immunohistochemical techniques are applied[5,16]. CD34 expression is generally lacking in PEComas.

Renal angiomyolipomas are frequently associated with TSC and even the sporadic cases present loss of heterozigosity (LOH) in chromosome 16p (harboring the *TSC2* locus)[3]. The pathogenesis of PEComas other than angiomyolipoma and LAM is not well understood. GISTs have not been reported to be associated with the TSC. *TSC2* LOH, as detected in some PEComas, have not been analysed in GISTs. Based on this and the finding that lesions of the PEComa family are KIT-negative in the majority of cases[5], it is likely that the coexistence of these two lesions in the current case represents a coincidence.

Hepatic PEComas, previously referred to as epithelioid or monotypic angiomyolipoma, are extremely rare[7,17-20]. To our knowledge, this is the first case reported in a patient with GIST.

In summary, we documented the first case of a GIST coexisting with a PEComa. Careful histopathological evaluation is mandatory for correct interpretation of the findings and, consequently, for appropriate patient treatment. A wide immunohistochemical panel is necessary to approach this differential diagnostic dilemma.

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CASE REPORT

Circumferential mucosal dissection and esophageal perforation in a patient with eosinophilic esophagitis

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Abstract

A young man with a previous history of episodes of mild solid food dysphagia was admitted with a total dysphagia. The esophagogastroduodenoscopy (EGDS) showed an extensive disruption of mucosal layer with a cul-de-sac in the lower part of the esophagus. Soon after the procedure, the patient suffered from an acute chest pain and subsequent CT scan demonstrated an intramural circumferential dissection of thoracic esophagus, and a mediastinal emphysema. An emergency right thoracotomy was performed, followed by a total esophagectomy with esophagogastroplasty and jejunostomy. The histopathology confirmed that mucosal and submucosal layers were circumferentially detached from muscular wall and showed an eosinophilic infiltration of the whole organ with necrosis and erosions of mucosal, submucosal and muscular layers. The diagnosis was esophageal perforation in eosinophilic esophagitis.

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Key words: Thoracic surgery emergency; Esophageal perforation; Eosinophilic esophagitis; Esophageal mucosal dissection; Esophagus surgery

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INTRODUCTION

Idiopathic eosinophilic esophagitis is an underdiagnosed disease with typical endoscopic findings. The clinical presentation includes solid food dysphagia in young adults who have an atopic predisposition. Food impaction necessitating endoscopic intervention is common. Mucosal rents and rarely perforations occur with simple passage of the endoscope[1]. We report a case of circumferential mucosal dissection in a young man with eosinophilic esophagitis.

CASE REPORT

A 32-year-old man was admitted into a local hospital service of gastroenterology with a diagnosis of food impaction. The patient had no previous history of upper gastrointestinal disease and had suffered from mild solid food dysphagia over the last years. All previous dysphagic episodes underwent spontaneous resolution and some of them were occasionally associated with various atopic symptoms. Upon admission, the patient was in good general conditions although presented mild distress due to tachypnea (RR 35/min); other vital signs were within normal limits. Since the patient complained of a total dysphagia in swallowing any liquids, it was impossible to perform a gastrografin esophagography. Therefore, the patient underwent EGDS that showed no food impaction, but an extensive disruption of mucosal layer with a cul-de-sac in the lower part of the esophagus. Soon after the endoscopy, the patient suffered from an acute chest pain and a severe discomfort with development of an extensive neck and anterior thoracic emphysema. A CT scan was performed.

The CT scan identified an intramural circumferential dissection of thoracic esophagus, a pneumomediastinum and a subcutaneous emphysema of the neck and of the anterior thoracic wall (Figure 1). Following the diagnosis of esophageal perforation, a right thoracotomy was performed. During the operation a 1.5 cm esophageal perforation at T1 level was detected. A peroperative EGDS was executed, and the mucosal and submucosal layers were circumferentially dissected from the muscular part of the esophageal wall. The dissection extended from 19 cm below the upper dental arcade up to the lower portion of the thoracic esophagus, ending at a cul-de-sac that stopped the endoscope progression. The mucosal and submucosal layers, circumferentially detached from muscular wall, looked like a whitish flap distally-fixed and floating inside the esophageal lumen (Figure 2). The endoscopy allowed to explore both the false and true visceral lumens. Due to the severe alteration of almost the whole thoracic esophagus wall, a simple suture of the perforation

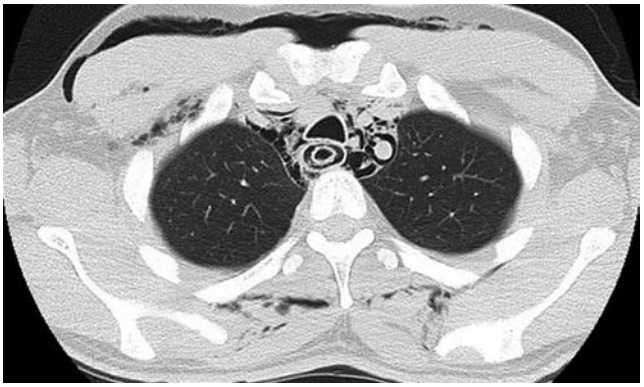


Figure 1 CT scan shows oesophageal double lumen associated with pmeumomediastinum and subcutaneous emphysema.

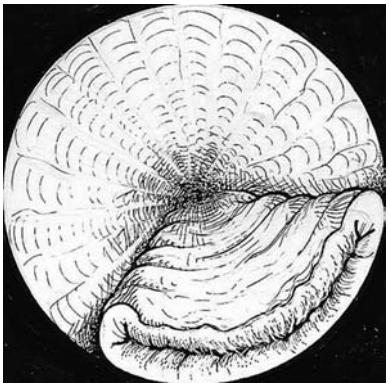


Figure 2 The drawing shows the endoscopic view of the esophagus: mucosal and submucosal layers are totally dissected from muscular wall. The false lumen ends in a cul de sac.

appeared hazardous, thus a total esophagectomy with esophagogastroplasty and jejunostomy was performed.

The histopathology confirmed an eosinophilic infiltration of the whole organ with necrosis and erosions of all mucosa, submucosa and in some areas of the muscular layer. The diagnosis was eosinophilic esophagitis.

The patient received 2 d of ICU. Gastrografin esophagogram on the 10th post- operative day showed normal pattern and motility of the neoesophagus. Three months after surgery, the patient had a good quality of life, ate a normal diet and did not experience any weight loss.

DISCUSSION

Eosinophilic esophagitis (EE) is an isolated, eosinophilic inflammation of the esophagus. The symptoms of EE used to be confused with gastroesophageal reflux (GERD).

Recent reports suggest a multi-step allergy process starting with an atopic skin response that primes the esophagus for further allergen exposure. A population of eosinophils different from gut and blood that specifically reaches the esophagus in EE was demonstrated[2]. The allergic reaction involved in EE is non-IgE mediated. The most commonly involved food includes milk, eggs, nuts, beef, wheat, fish, shellfish, corn and soy. However, almost all the food is implicated[3].

The diagnosis of EE is suggested by an unexplained dysphagia or by a food impaction in a young adult with a personal and family allergic history.

Clinical features include dysphagia (93%), food impaction (62%), heartburn (23.6%) and history of atopia (51.6%)[4]. Endoscopic findings are mucosal fragility/oedema, rings, strictures, whitish pinpoint exudates and small-calibre esophagus in various cases whereas 8.8% of patients have a normal endoscopy[4]. The presence of more than 15 eosinophils per high power field on esophageal biopsies is required to establish the diagnosis[4]. Therapeutic options include restriction of diets, various medicaments (systemic and topical corticosteroids, leukotriene receptor antagonists and, most recently, an anti-interleukin-5 monoclonal antibody) and endoscopic dilatation[5]. The administration of corticosteroids resulted in symptomatic improvement in more than 95% of cases. A trial with corticosteroids before bougienage may reduce the active inflammation and the complication rate[2]. Esophageal perforation is described as a complication[4] and one case was treated with subtotal esophagectomy[6]. Although a spontaneous circumferential mucosal dissection was already described[7], we are not aware of any other cases associated with eosinophilic esophagitis.

We believe that in our patient the dissection is most likely spontaneous, since no impacted food has been found in either the endoscopic examination or the resected esophagus. However, we can not exclude that the dissection may be caused or enlarged by the esophagoscopy, therefore this risk must be always born in mind while deciding to perform an endoscopy for young patients with sudden total dysphagia and with a personal and familial history of atopic symptoms. With regard to esophageal perforation, it is certainly iatrogenic, since the symptoms correlated with this complication (acute chest pain, severe discomfort, extensive neck and anterior thoracic emphysema) appeared at the end of the endoscopic examination.

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CASE REPORT

Levovist ultrasonography imaging in intracystic hemorrhage of simple liver cyst

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Abstract

The differential diagnosis between intracystic hemorrhage and cystadenocarcinoma of the liver is often difficult even with the use of various imaging modalities. A 73-year-old woman was admitted to our hospital with the complaint of right upper quadrant pain. Ultrasonography (US) demonstrated a heterogeneous echogenic cyst measuring 11 cm × 8 cm in size in S2 of the liver, indicated intracystic hemorrhage of simple liver cyst or cystadenocarcinoma, but the differential diagnosis was considerably difficult. Levovist (Schering, Berlin, Germany) US revealed no enhancement of the intracystic structures, suggesting a clot in the case of intracystic hemorrhage. An operation was performed and the resected lesion showed a solitary benign liver cyst, measuring 5.5 cm × 4.7 cm × 8.5 cm containing a large blood clot. The patient had an uneventful recovery after the surgery. Levovist US may play an important role in discrimination between intracystic hemorrhage of simple hepatic cysts and cystadenocarcinoma of the liver.

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Key words: Liver cyst; Cystadenocarcinoma; Simple cyst; Galactose acid palmitique; Levovist

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INTRODUCTION

Hemorrhage into a simple liver cyst is rather uncommon. The differential diagnosis between intracystic hemorrhage and cystadenocarcinoma of the liver is difficult with the various imaging studies currently available[1,2]. Consequently, hepatectomy has been performed unnecessarily, even in cases with benign liver cysts with intracystic bleeding, based on a wrong preoperative diagnosis of cystadenocarcinoma. We report on a patient with intracystic hemorrhage complicating a simple liver cyst in whom the diagnosis was made accurately by Levovist (Schering, Berlin, Germany) Ultrasonography (US) Imaging.

CASE REPORT

A 73-year-old woman with chronic hepatitis C had been under follow-up for two years. She had been followed up by laboratory examination every month and imaging studies such as US and Computed Tomography (CT) every six months as screening procedures for hepatocellular carcinoma. The previous US and CT had only shown a simple cyst measuring 1.3 cm in diameter in segment 4 (S4) of the left lobe. The patient did not complain of any abdominal discomfort during this period, nor was there any history of abdominal injury.

The patient was admitted to hospital with the complaint of right upper quadrant pain. Her blood pressure was 110/62 mmHg, pulse was 84/min, and physical examination revealed spontaneous pain, tenderness, and no muscular defense in the abdomen. The liver was not palpable under the right rib cage. The laboratory examination findings were almost normal



Figure 1 US image showing a heterogeneous echogenic cyst in S2, measuring 11.0 cm × 8.0 cm and showing intracystic septations and structures.

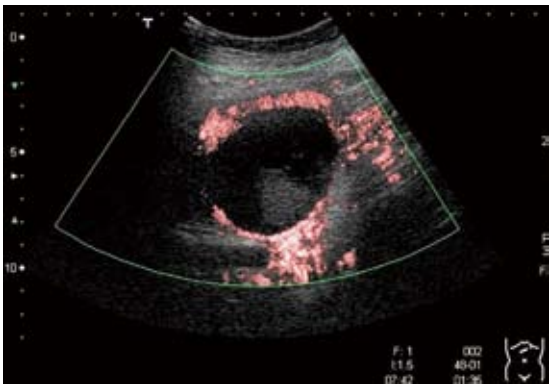


Figure 2 CEU images demonstrating absence of enhancement of the intracystic septations and structures and smooth of the enhanced cyst wall.

except as follows: anemia (red blood cell count, 399 × 10^4/mm^3; hematocrit, 30%; hemoglobin, 12.6 g/dL) and elevated serum CRP (3.5 mg/dL). The serum was negative for antibody against Echinococcus antigens by enzyme-linked immunosorbent assay.

US demonstrated a heterogeneous echogenic cyst measuring 11 cm × 8 cm in size with intracystic structures in S2 of the liver (Figure 1), indicating a intracystic hemorrhage of simple liver cyst or cystadenocarcinoma, but the differential diagnosis was considerably difficult on the US imaging. Levovist (Schering, Berlin, Germany) US revealed no enhancement of the intracystic septations and structures, suggested a clot in case of intracystic hemorrhage. Moreover the enhanced cyst wall was smooth in contour, suggested a simple cyst (Figure 2). Hence, the hemorrhage into a simple cyst including intracystic blood clot was indicated by Levovist US. On plain CT, the intracyst showed heterogeneous low-density, and intracystic septations were not detected. On magnetic resonance imaging (MRI), T1-weighted images revealed the clot as a low-intensity and the fluid as a high-intensity, whereas T2-weighted image shows the clot as low-intensity and the fluid as high-intensity. Although a high-intensity lesion on T1-weighted images is not specific for hemorrhage and can be observed in other conditions (mucinous fluid, melanin component, and fat content), the visualization of a high-intensity lesion on T2-weighted images may suggest

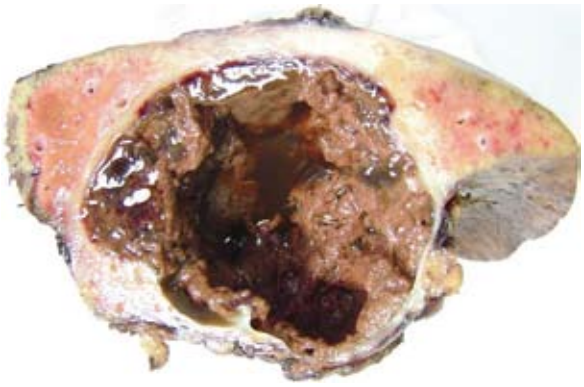


Figure 3 Histological examination showed that the intracystic structures were clots caused by the intracystic hemorrhage.

the diagnosis of intracystic hemorrhage. No contrast agent could be used both in CT and MRI studies, because of the patient's history of asthma. Based on the imaging findings, we diagnosed the case as one of hemorrhage into a simple cyst. Because of the risk of the enlargement and rupture of the cystic lesion, operation was considered to be indicated. Lateral sectionectomy, cholecystectomy, and intraoperative cholangio-graphy (IOC) were performed. Exploratory laparotomy revealed a large cystic lesion which occupied the left lobe of the liver with adhesion of the omentum minor to its wall. IOC revealed no communication between the intrahepatic bile duct and the cystic lesion. The resected lesion showed a solitary benign liver cyst, measuring 5.5 cm × 4.7 cm × 8.5 cm containing a large blood clot (Figure 3). The blood clot in the cyst was apparently visualized as the intracystic structures on the US and MRI and CT. The cystic wall consisted of fibrous tissue with capillary proliferation and hemorrhage, and was lined by one row of cuboidal epithelial cells. The patient had an uneventful recovery and was discharged 17 d after the surgery.

DISCUSSION

Cystic lesions in the liver are currently classified into acquired and congenital cysts, or parasitic and nonparasitic cysts. Acquired cysts include traumatic, inflammatory, and proliferative cysts[3]. Simple cysts or nonparasitic liver cysts are considered to be congenital and most simple cysts encountered in clinical practice are congenital. The etiology of simple cysts is unclear, and they presumably arise from developmental defects during the formation of bile ducts. The lining epithelium of these cysts is identical to that of the biliary tract[4], although an open communication with the biliary system is unusual.

Hepatic simple cysts are predominantly asymptomatic. However, symptoms may be caused by rapid growth or occurrence of complications. The complication rate has been reported to be about 10%, and complications include intracystic hemorrhage, infection, intraperitoneal rupture, torsion, inflammation, and obstructive jaundice, with hemorrhage and infection as the most commonly encountered complications[5].

In our case, the differential diagnosis would have

been even more difficult if a cystadenocarcinoma was complicated by intracystic hemorrhage. One of the characteristic findings of cystadenocarcinoma on sonography has been reported to be highly echogenic internal septations[6].

In many countries, microbubble contrast agents are now available for improved US imaging. Levovist is one of such microbubble agents, consisting of an admixture of galactose granules with a small (0.1%) amount of palmitic acid that produces multiple small (approximately 3 μm) stabilized air bubbles when suspended in water. Levovist produces Doppler enhancement of the blood pool for 2-5 min[7], followed by a late hepatosplenic phase that lasts for more than 30 min[8]. In our patient, the intracystic structures mimicking malignancy that was visualized on US was demonstrated to be an avascular lesion on CEU, suggested a clot of intracystic hemorrhage. In addition, the enhanced cyst wall was shown to have a smooth contour, suggested the diagnosis of a simple cyst. Hence, we diagnosed the hemorrhage into a simple cyst.

The treatment of a simple liver cyst complicated by intracystic hemorrhage can be either surgical or nonsurgical. In recent years, there has been a trend away from surgical treatment, as injection of absolute alcohol into the cyst has been shown to yield better results with fewer complications. However, in our patient, surgery was chosen because the cyst began to grow rapidly in size, with the attendant risk of rupture. Therefore, we could examine the resected tissue specimens which showed that the lesion was an encapsulated unilobular cyst filled with morphologically irregular blood clots. The histological findings in resected tissue specimens in relation to the MR images of intracystic hemorrhage in simple liver cysts has previously been reported by one group[9]. In our reported case, we could compare the histological findings in the resected tissue specimens with the CEU findings. Moreover, some groups have even reported detection of the active bleeding point in such cases by CEU[10,11]. In our case, the active bleeding point was not detected, perhaps because the bleeding had already stopped. However, CEU may be useful for diagnosing active intracystic hemorrhage.

In conclusion, CEU may play an important role in the correct diagnosis of a simple hemorrhagic cyst by demonstrating the avascularity of the visualized intracystic structures. We followed up the patient of a simple liver cyst complicated by intracystic hemorrhage in whom CEU was very useful for diagnosis.

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CASE REPORT

Mycobacterium ulcerans infection as a cause of chronic diarrhea in an AIDS patient: A case report

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Abstract

Chronic diarrhea is one of the most frequent gastrointestinal manifestations in acquired immunodeficiency syndrome (AIDS). Protozoa and nontuberculous mycobacteria (NTM) are opportunistic pathogens that can easily infect these patients. Among the NTM, *Mycobacterium avium* complex (MAC) is the most frequently observed pathogen in HIV-infected patients. However, NTMs other than MAC have not been reported as a gastrointestinal pathogen as yet. We present a case of chronic diarrhea in an AIDS patient in whom *Mycobacterium ulcerans* and *cryptosporidium* co-infection is evidenced from colonic tissue.

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Key words: *Mycobacterium ulcerans*; *Cryptosporidium*; HIV; Chronic diarrhea

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INTRODUCTION

HIV infection, which frequently involves the gastrointestinal tract, may be associated with diarrhea, abdominal pain, esophageal symptoms, malabsorption, and weight loss[1,2]. The gastrointestinal tract is the major target for pathogens in HIV-infected patients and as many as a third of patients with AIDS are initially seen by a gastroenterologist[3]. In those instances, diarrhea is the most frequent manifestation in AIDS and HIV-positive patients. Diarrhea has been reported to be present at the same time during their illness in as many as 80% of North American patients with AIDS, and the prevalence may approach to 100% in developing countries, causing a significant morbidity and mortality[4-6]. Those suffering from chronic diarrhea tend to have a low quality of life and seem to have higher annual health costs. Typical infectious diseases affecting the intestinal tract in HIV-infected patients are caused by protozoa (*Microsporidium, Cryptosporidium*), bacteria (*Clostridium difficile, Salmonella, Shigella, Campylobacter*, and MAC), viruses (Cytomegalovirus, Herpes simplex) and fungi (*Candida species*). Before the HIV epidemic, NTM was an unusual cause of disease in humans. Nowadays, however, NTM especially MAC, is one of the most common causes of intestinal bacterial infection in AIDS patients[2,7]. But non-MAC NTMs, including *Mycobacterium ulcerans*, have not been reported as a pathogen in the gastrointestinal tract.

We report herein a case of colonic infection with an unusual NTM, *Mycobacterium ulcerans*, which caused chronic diarrhea in a patient with AIDS.

CASE REPORT

A 40-year-old man complained of persistent diarrhea associated with a 9 kg weight loss during the last 3 mo. He described his diarrhea as watery stools that occurred five to six times per day, especially at night. There was no previous history of travel, food, medication, or any medical or familial disease. He denied melena or hematochezia. On physical examination, the patient had a chronic ill-looking appearance but there were no remarkable findings except for a slightly dehydrated tongue. His blood pressure was 110/70 mmHg, pulse was 70 beats per minute, respiratory rate was 20 breaths per minute, and temperature was 36.2℃. His height was 167 cm and weight was 54 kg. The abdomen was soft and nontender

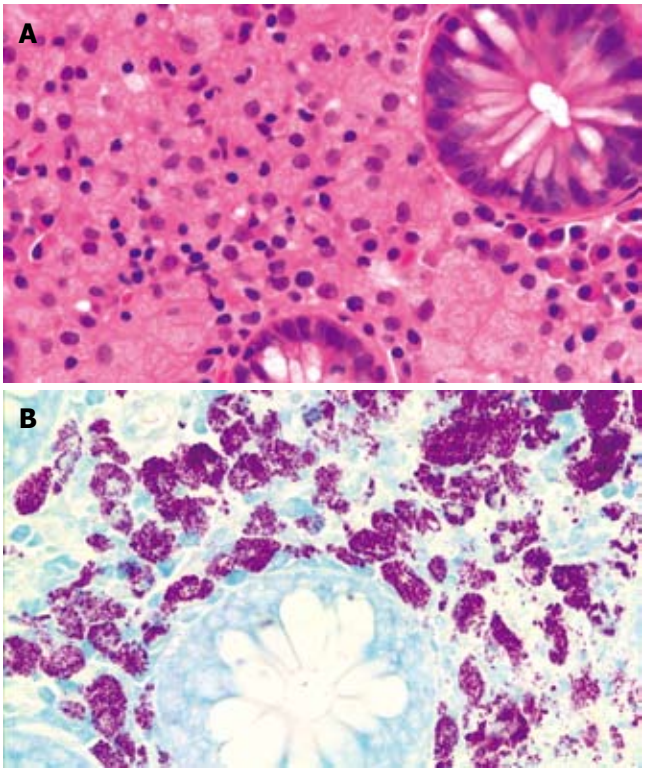


Figure 1 Pathologic examination of biopsy specimen from sigmoid colon revealing foamy histiocytic collections in lamina propria by hematoxylin and eosion stain (**A** × 400) and innumerable acid-fast bacilli collections by Ziehl-Neelsen stain (**B** × 400).

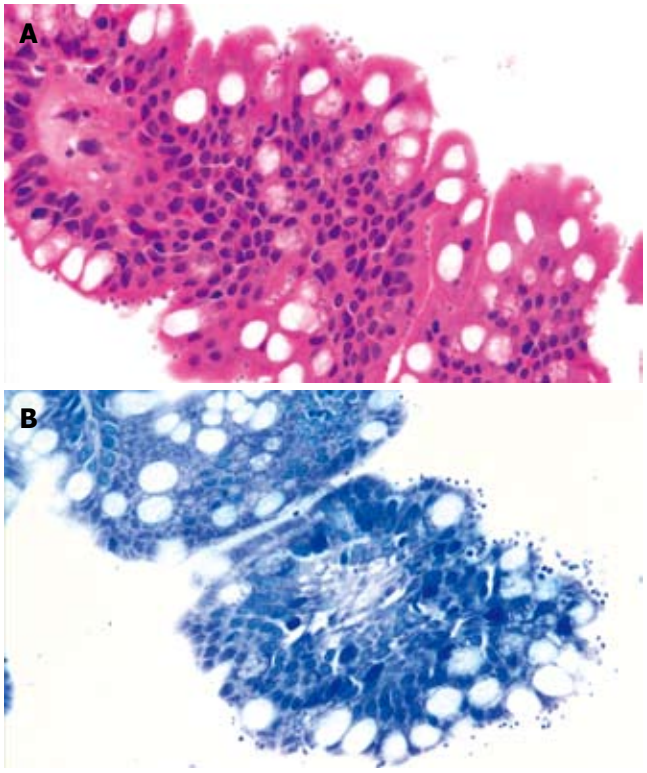


Figure 2 Pathologic examination of biopsy specimen from terminal ileum showing many tiny organisms of the *Cryptosporidium* species with mild chronic inflammation on the surface of the luminal border of the crypt epithelial cells by hematoxylin and eosion stain (**A** × 400) and Giemsa stain (**B** × 400).

without hepatosplenomegaly. The bowel sound was slightly increased. Rectal examination revealed no palpable masses or blood clots. The hemoglobin level was 14.4 g/dL, white blood cell count was 4200/mm^3, and platelet count was 159 000/mm^3. The serum electrolyte levels, urinalysis, and tests of renal, liver, and thyroid function were normal. The C-reactive protein level was 0.1 mg/dL. Stool examinations for ova, parasites, bacterial pathogens, and *Clostridium difficile* toxin were negative. Colonoscopy showed normal findings but a blind mucosal biopsy was done at the terminal ileum and sigmoid colon to evaluate the chronic diarrhea. Also, anti-HIV antibody was checked to rule out AIDS, which is an important cause of chronic diarrhea. The result of anti-HIV antibody was positive. CD4 T cell count was 168/mm^3. We asked the patient about his medical history again and he admitted to an HIV positive history to us. He was diagnosed with AIDS at another hospital in 2002 and treated with highly active antiretroviral therapy (HAART). However, he voluntarily discontinued his treatment in 2004. The biopsy specimen from the sigmoid colon demonstrated foamy histiocytic collections in lamina propria by hematoxylin and eosin stain and revealed innumerable acid-fast bacilli collections by Ziehl-Neelsen stain (Figure 1). The possibility of NTM on colonic biopsy would have to be considered instead of *Mycobacterium tuberculosis* because of innumerable acid-fast bacilli collections. The biopsy specimen from the terminal ileum demonstrated mild chronic inflammation with protozoa infection consistent with cryptosporidiosis (Figure 2). To further evaluate the small bowel, a capsule endoscopy was performed. The whole small bowel showed mild edematous and hyperemic mucosal change (Figure 3).

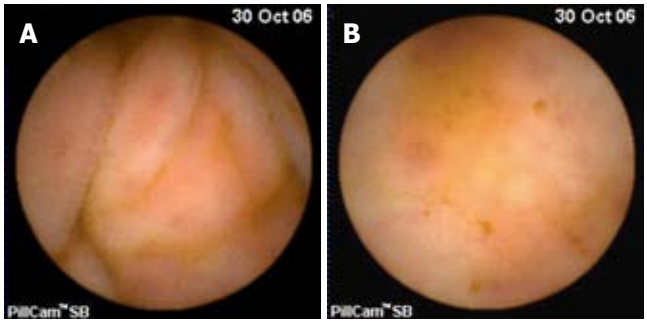


Figure 3 Capsule endoscopic findings of small bowel showing mild edematous (**A**) and hyperemic (**B**) mucosal change.

PCR-restriction fragment length polymorphism analysis (PRA)

To identify the species of NTM, we used PCR-restriction fragment length polymorphism of the rpoB gene. PRA offers an easy, rapid, and inexpensive way to identify *Mycobacterium* species in a single experiment. Recently, Lee *et al*[8] chose the rpoB gene as a target and the restriction analysis of a 360-bp fragment within the rpoB gene after single Msp I digestion showed that it was highly effective for differentiating most *Mycobacterium*. They suggested that PRA using the novel region of the rpoB gene is more simple, rapid, and accurate versus other

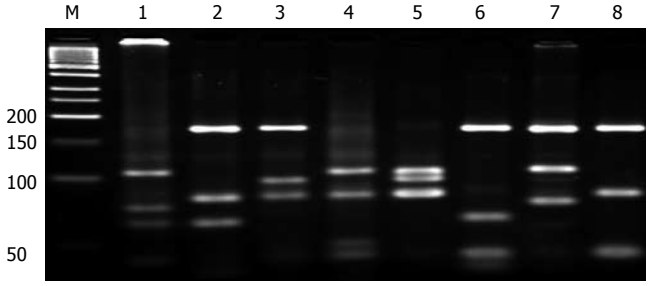


Figure 4 Identification of the species of NTM by PCR-restriction fragment length polymorphism of the rpoB gene. Lane: M, DNA size marker; lane1, M. ulcerans; lane 2, *M. tuberculosis*; lane 3, *M. intracellulare*; lane 4, *M. avium*; lane 5, *M. abscessus*; lame 6, *M. kansasii*; lane 7, *M. fortuitum*; lane 8, *M. gordonae*.

conventional methods such as chromatography or DNA sequence analysis. Briefly, we got the genomic DNA from paraffin-embedded colonic specimen. PCR was carried out with 10 ng genomic DNA, 10 pmol of each primer, 5'-TCAAGGAGAAGCGCTACGA-3' (RPO5') and 5'-GGATGTTGATCAGGGTCTGC-3' (RPO3') and DyNAzyme Ⅱ DNA polymerase (FINNZYMESY, Espoo, Finland) resulted in a 360-bp PCR product. After successful amplification, the PCR products were subjected to restriction enzyme digestion with 5U of Msp I (Boehringer Mannheim Biochemicals, Mannheim, Germany). For interpreting the PRA profile generated by each species, a 50-bp ladder DNA size marker (Boehringer Mannheim) was used. From the PRA, we discovered that the NTM species was *Mycobacterium ulcerans* (Figure 4).

The patient was diagnosed with *Mycobacterium ulcerans* and *cryptosporidium* co-infection. He was treated with clarithromycin and ethambutol to target the NTM and with HAART for underlying AIDS. After three months of treatment for NTM, the patient said that his diarrhea was much improved.

DISCUSSION

Since the discovery of AIDS in 1981, HIV infection has spread quickly and become a very serious health problem for humans, not only in Western world, but also in Asian area. Since the first case of HIV infection was reported in Korea at 1985, the number of those infected with HIV has steadily increased. Despite the introduction of HAART, diarrhea remains a common disease manifestation related to HIV infection and is a diagnostic problem. The incidence of chronic diarrhea in AIDS patients does not change after HAART[9]. In other words, the incidence of opportunistic infections is still too high to ignore them. Opportunistic infections are responsible for 68%-85% of diarrhea in HIV-infected patients[7,10].

Initially, we did not know the exact medical history of the patient. However, HIV-related diarrhea must be included in evaluating chronic diarrhea, especially when it is associated with weight loss. Therefore, we performed a routine serologic test for HIV infection. Patients with chronic diarrhea in HIV-infected persons often cause a difficult diagnostic problem. There are proved pathogens or causes of drugs including protease inhibitors, but no causes of diarrhea are commonly encountered. The usefulness of endoscope in evaluating diarrhea in HIV-infected patients has been well investigated[11-13]. Because of the common involvement of the small bowel, colonoscopy is preferred to flexible sigmoidoscopy in HIV-infected patients with chronic, unexplained diarrhea[13]. Although colonoscopy is frequently performed in these patients, the usefulness of biopsy of normal mucosa is uncertain. Shah *et al*[14] suggested that colonoscopy with biopsy is a useful tool in the investigation of patients with chronic diarrhea, which yields a histological diagnosis in 31% of patients. In our case, we performed a blind biopsy from normal mucosa, because the serology for HIV was positive, and we thought that the histological evaluation would give us good information for the proper diagnosis. With the histological diagnosis, we had a clue as to how to properly treat the patient. This is in accordance with the suggestion of Shah *et al*[14] that colonoscopy with biopsy is a useful tool in the investigation of patients with chronic diarrhea, not only from the abnormal mucosal lesion but also from the normal one.

NTMs are ubiquitous organisms that are different from tuberculosis and represented by more than 65 different species[15]. The most frequent forms of NTM include *Mycobacterium avium* complex (MAC, 61%), *Mycobacterium fortuitum* (19%) and *Mycobacterium kansasii* (10%), with smaller percentages of *Mycobacterium gordonae* and *Mycobacterium chelonae*[15,16]. Mycobacterial involvement of the bowel either by tuberculosis or MAC may lead to diarrhea. Antony *et al*[17] reported that MAC is the most commonly identified organism in patients with chronic diarrhea and low CD4 lymphocyte counts. Although tuberculosis appears to be symptomatic in all cases, a large number of patients with MAC have an asymptomatic infection of the gut. Endoscopic findings of MAC infection may be nonspecific. Sun *et al*[18] reviewed 55 reported cases of MAC infection with an endoscopic description which involved the gastrointestinal tract in AIDS patients, and found that the most common endoscopic finding is multiple raised nodules (38%) in the involved sites. The other endoscopic findings of MAC infection include normal findings (36%), erythemas (13%), ulcerations (11%), edemas (11%), friabilities (11%), reduced mucosal vascular patterns (9%), erosions (4%), confluent nodules (4%), strictures (2%), and aphthous erosions (2%). Therefore, for a definitive diagnosis in symptomatic patients, especially those without typical endoscopic findings, it is essential to obtain tissue specimens for histopathologic examination and microbiologic culture. Isolates of *Mycobacterium* from tissue biopsies or blood are almost always indicative of the disease[19]. The organism is readily seen on biopsy specimens with acid-fast staining, and the number of organisms is often striking. Rarely, a NTM other than a MAC has been isolated upon a colonic biopsy in a HIV patient[19]. In our case, a very rare NTM, *Mycobacterium ulcerans*, was isolated in colonic mucosa, and it is presumed to be the cause for the patient's chronic diarrhea. Many clinical syndromes are similar to those seen with MAC and only can be differentiated by identification of the organism, like PRA or DNA sequence analysis[20].

Mycobacterium ulcerans is known to cause skin diseases, such as Buruli ulcer, an important health problem in

several West African countries. Although the disease is known to be associated with contaminated water, the mode of transmission has not yet been understood[21,22]. *Mycobacterium ulcerans* produces the toxin mycolactone, which induces necrosis and ulceration by its own cytotoxic and immunosuppressive properties. HIV infection has not been reported as a risk factor for Buruli ulcer[23]. However, severe clinical forms of the disease have been described in HIV-positive patients[24]. To our knowledge, this is the first case of colonic infection with *Mycobacterium ulcerans*, which caused chronic diarrhea in a patient with AIDS.

In conclusion, *Mycobacterium ulcerans* and *crypto-sporidium* co-infection is the cause of chronic diarrhea in AIDS patients. Serologic test of HIV must be performed when evaluating chronic diarrhea, especially when it is associated with significant weight loss. We think that colonoscopy with routine biopsy is a useful tool for accurate diagnosis of chronic diarrhea.

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CASE REPORT

Coincidence of hepatocelluar carcinoma and hepatic angiomyolipomas in tuberous sclerosis complex: A case report

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Abstract

Tuberous sclerosis complex (TSC) is a dominantly inherited disorder which characterized by the growth of hamartomatous in multiple organs. Unlike the common development of renal angiomyolipoma, hepatic angiomyolipoma rarely occur in patients with TSC. We report here a patient with hepatic angiomyolipomas and concurrent hepatocellular carcinoma in TSC. This represents the first reported case in English literature. In this patient, multiple hepatic angiomyolipomas were diagnosed with recognition of their fat components and typical clinical settings. Hepatocellular carcinoma in the left liver lobe was definitely diagnosed by US guided biopsy. In such clinical settings, fat containing lesions in liver can be reasonably treated as angiomyolipomas, but non fat containing lesions must be differentiated from hepatocellular carcinoma, imaging guided biopsy can be adopted to confirm the diagnosis.

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Key words: Angiomyolipoma; Liver; Tuberous sclerosis complex; X-ray computed tomography; Ultrasonography

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INTRODUCTION

Tuberous sclerosis complex (TSC) is a dominantly inherited disorder with a prevalence of 1/6000 to 1/9000 in general population[1,2]. In patients with TSC, the incidence of renal angiomyolipoma has been reported in up to 80%, but hepatic angiomyolipoma has been less reported[3,4]. We recently encountered a patient with pathologic proved coincident hepatocellular carcinoma and hepatic angiomyolipomas in TSC. To our knowledge, there is no reported case of hepatic angiomyolipomas with concomitant hepatocellular carcinoma in TSC in English literature. We present here the imaging findings of this case, and discuss the diagnosis and it's practical significance.

CASE REPORT

A 51 years old woman was admitted in our hospital because of intrahepatic nodules found incidentally by abdominal ultrasonography (US) on general health examination. Her past medical history showed a right nephrectomy for renal angiomyolipomas 16 years prior to this admission. Physical examination revealed multiple miliary russet papilla on her face and several periungual fibroma. A palpable mass was touched with mild knock pain in the left flank. Laboratory analysis showed normal liver function, hepatitis B surface antigen (HBsAg) was positive. Concentration of serum alpha fetoprotein (αFP) was 7694 μg/mL (normal, < 20 μg/mL). Urine routine test and renal function were normal.

Abdominal US revealed multiple well-circumscribed nodules of various size with homogeneous hyperechoic nodules in the liver, and a hypoechoic nodule in the left liver lobe was noted. Plain helical CT scan showed multiple round well-defined nodules with variable fatty density in the liver (Figure 1A). After administration of intravenous contrast material, the non-fat components of these lesions were enhanced heterogeneously (Figure 1B). Apart from those fat containing lesions, the precontrast CT scan demonstrated a poor defined hypodensity mass with no fat component situated in the left liver lobe (Figure 1C). On dynamic contrast enhanced CT, it presented as nearly homogeneous isodensity in the arterial phase, hypodensity with dotted vessel in the portal venous phase in comparison with the surrounding liver (Figure 1D and E). On coronal reconstruction images, it was adjacent to the left portal vein. Left giant renal heterogeneous mass with fatty density and hemorrhage was found meanwhile (Figure 1F).

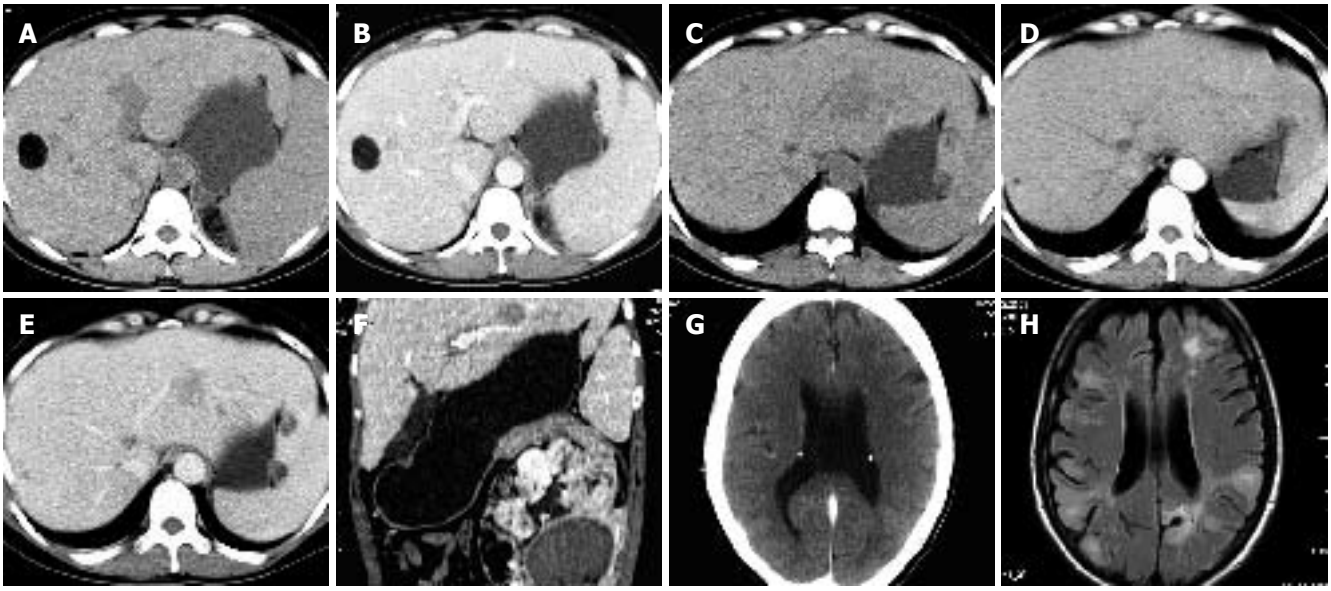


Figure 1 Photocopy detect of the patient. **A**: Plain helical CT scan demonstrats a round well defined nodule with fat component in the liver; **B**: Enhanced CT scan shows that the non-fat component of the lesion is mildly enhanced; **C**: Plain CT scan shows an entire solid mass with no fat component in the left liver lobe and multiple distributed nodules with variable fat components in the liver; **D**: Enhanced CT shows the solid mass with no fat component in the left liver lobe as nearly homogeneous isodensity in the arterial phase; **E**: Enhanced CT scan shows the solid mass in the left liver lobe as hypodensity with dotted vessel in the portal venous phase; **F**: Coronal reconstruction images shows that the mass is adjacent to the left portal vein, and a left giant renal heterogeneous mass with fatty density and hemorrhage is noted; **G**: Cerebral CT scan demonstrats symmetrical subependymal calcified tuber; **H**: FLAIR MR imaging shows multiple cortical tubers with high signal intensity in the bilateral hemisphere, linear and irregular spherical high signal areas from periventricular to subcortical regions in the left occipital and frontal lobes.

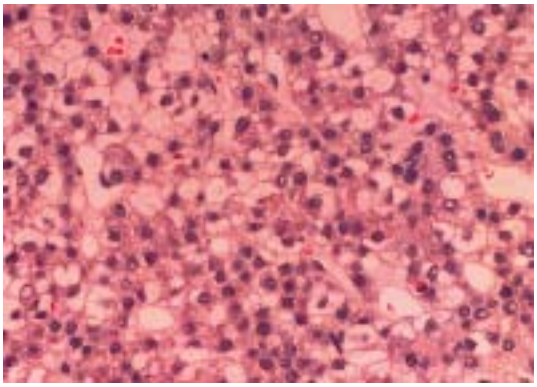


Figure 2 High-power photomicrograph shows tumor cells with eosinophilic cytoplasm arranged in solid pattern and inconspicuous sinusoid-like or slit-like blood vessels (HE, original magnification × 100).

Based on the abdominal imaging findings and the history of right nephrectomy for renal angiomyolipoma, TSC with multiple organs involved was suggested. Then cerebral CT scan was applied immediately after enhanced abdominal CT scan. Cerebral CT scan demonstrated symmetrical subependymal calcified tuber (Figure 1G). MRI showed multiple cortical tubers with high signal intensity in the bilateral hemisphere, linear and irregular spherical high signal areas from periventricular to subcortical regions in the left occipital and frontal lobes on T2WI and FLAIR sequence (Figure 1H). According to the typical clinical information and imaging findings, TSC with multiple organs involved, multiple angiomyolipomas in liver and left kidney was first considered. But the possibility of concurrent hepatocellular carcinoma can't be excluded in this patient because of the remarkable increasing concentration of serum alpha fetoprotein (7694 μg/mL).

Ultrasound-guided biopsy of the mass with no fat component in the left liver lobe with 18-gauge needle was performed. Histopathological examination of the specimen showed heptocellular carcinoma. Neoplastic cells with eosinophilic cytoplasm were arranged in solid pattern, and inconspicuous sinusoid-like or slit-like blood vessels were noted (Figure 2). Immunohistochemical study showed AFP+, CD34+, HbsAg and HMB45 were negative.

The patient refused to undergo liver resection; ultrasound-guided radiofrequency ablation was performed. CT scan showed complete ablation of the heptocellular carcinoma, and she was uneventful during one year follow up.

DISCUSSION

TSC is an autosomal dominant disorder involving multiple organs[1,2]. The mutation of two tumor suppressor genes (TSC1 and TSC2) could account for the pathogenesis of hamartomatous in tuberous sclerosis[5]. In recent literature, hepatic angiomyolipoma has been reported in up to 13%-21% of patients with TSC, especially in female patients with bilateral diffuse renal angiomyolipomas[6]. However, coexistence of hepatocellular carcinoma and hepatic angiomyolipoma is extremely rare [7]. To our knowledge, no report of coincident hepatocellular carcinoma and hepatic angiomyolipomas in TSC exist in English literature.

Angiomyolipoma is a rare benign, non-encapsulated mesenchymal tumor consisting of vessels, smooth muscle and adipose tissue[3]. Hepatic angiomyolipoma is

often asymptomatic and found incidentally on imaging studies[6]. In the setting of tuberous sclerosis, hepatic angimyolipomas are often accompanied by bilateral renal angiomyolipomas. Imaging studies play a key role in the diagnosis of hepatic angimyolipoma. On ultrasonography, hepatic angiomyolipomas often presented as multiple well defined, round hyperechoic nodules. Plain CT scan showed them as multiple hypodensity nodules with variable amounts of fatty density. After administration of intravenous contrast material, the non-fat components of these lesions were enhanced heterogeneously. The imaging findings of hepatic angiomyolipoma are similar to those of renal angiomyolipoma. The diagnosis of hepatic angiomyolipoma is often made on recognition of its fat component[6-9].

However, nearly 50% hepatic angiomyolipomas may present as solid mass with least or no fat-component[10]. Plain CT scan usually shows it as well circumscribed, hypodensity nodules. On dynamic contrast CT, it is often heterogeneous hyperdense in the arterial phase, hypodense with prominent central vessel in the portal venous phase. The enhancement pattern of hepatic angiomyolipoma is similar to that of hepatocellular carcinoma, so it's difficult to be differentiated from hepatocellular carcinoma[9-11]. However, no case of concurrent hepatocellular carcinoma and hepatic angiomyolipoma in TSC has been previously reported in the literature. It has even been reported that non-fatty hepatic lesions in TSC can be regarded as hepatic angimyolipomas[6,11]. In our patient, the imaging findings of multiple hepatic angiomyolpomas with fat component were in accordance with previously reported. The diagnosis of TSC with multiple organs involved was definitely based on the revised clinical diagnostic criteria[1]. But hepatocellular carcinoma can not be excluded in this patient, because the solid mass in the left liver lobe was presented as poor defined and hypovascular, and no fatty density can be found in it on the dynamic contrast enhanced CT. The imaging findings of the solid mass were not in accordance with those of hepatic angiomyolipomas without fat components. In addition, the markedly increasing concentration of serum alpha-fetoprotein strongly suggested the existence of hepatocellular carcinoma. This was confirmed with ultrasound guided biopsy of the solid mass with no fat component in the left liver lobe. Our case suggests that non-fatty hepatic lesions in TSC can't be regarded as angiomyolipomas arbitrarily, they must be differentiated from hepatocellular carcinoma, because the natural history of hepatic angiomyolipoma is benign, and it needn't surgery, except for suffer of complications[12], but hepatocellular carcinoma should be removed surgically.

In conclusion, awareness of coincident hepatocellular carcinoma and hepatic angiomyolipomas in TSC is important. When intrahepatic mass with no fat component be found in such clinical setting, the possibility of concomitant hepatocellular carcinoma and hepatic angiomyolipoma should be considered. Imaging studies and laboratory tests can help in the differentiated diagnosis. Imaging guided biopsy can be adopted to confirm the diagnosis.

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Use of chorioamniotic membrane instead of bogota bag in open abdomen: How I Do It?

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Abstract

It is one of the most important problems for general surgeons to decide which operation should be undertaken on patients with intra-abdominal infection, especially those with concomitant abdominal hypertension. Recentlly, closure techniques using prosthetic meshes in order to retain abdominal tension and to control sepsis have become very popular for patients with abdominal sepsis and hypertension. We used chorioamniotic membrane instead of plastic material to cover the open abdomen. We conclude that human chorioamniotic membrane prepared under sterile conditions may be an alternative to conventional plastic bags in daily practice, for preventing serosal erosion and fistulas in patients undergoing open abdominal surgery.

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Key words: Intraabdominal sepsis; Chorioamniotic membrane; Bogota bag

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TO THE EDITOR

There are some important indications for open abdominal surgery, such as abdominal compartment syndrome, damage control surgery, severe peritonitis and infected pancreatic necrosis. A Bogota bag, which is sutured to the abdominal wall or to the fascia, is used to cover the open abdomen. It needs to be changed frequently until the underlying problem in the abdomen is solved. If there is no need of early abdominal closure, a skin graft is placed over the granulation tissue that covers the viscus. After that, the deliberately created ventral hernia is repaired 6-12 mo later. The above situation has been changed considerably with the introduction of vacuum application and biological prosthetic material[1-5]. Until recently, the practice was to discharge patients with an open fascia and partially closed skin.

An open abdomen is a heavy burden for the body. Electrolyte-fluid losses and protein-energy requirements are high in the open abdomen. The need for intensive care also increases costs, and the condition becomes more serious if there are coexisting fistulae or an open stoma in the gut.

We consider the use of chorioamniotic membrane as a biological material when vacuum application is not possible or plastic cover might be harmful. To the best of our knowledge, this is the first instance in which chorioamniotic membrane has been used for this purpose.

The chorioamniotic membrane was obtained from elective cesarean section, after patient consent was obtained. The membrane was prepared under sterile conditions by removing the cotyledons from the placenta (Figure 1). The membrane was then kept in 1 L normal saline containing 1 g streptomycin and 1 MU penicillin at 4℃ for 24 h. After each irrigation of the abdomen, chorioamniotic membrane was applied as a cover, with maternal surface facing the viscera (Figure 2). This procedure was repeated every 2 d. Immediately following the second application, it was clearly observed that serosal erosion showed signs of healing and the adhesions between the loops became clearer. The application decreased edema of the gut and abdominal wall, and the bowel was attached to the abdominal wall (Figure 3). Patients were discharged for definitive treatment planned after 6 mo. There were no problems in early follow-up. Six months postoperatively, ventral hernias were successfully repaired using omental or online mesh hernioplasty.

Open abdomen is a life saving procedure when indicated. Its mortality and morbidity increase when combined with that of the primary underlying illness[5]. Currently, patients with open abdomen are discharged with a ventral hernia, which is covered with a graft that needs further repair.

It is reported that by using vacuum methods, fascial defects are reduced sufficiently to be closed[1-4]. It is also

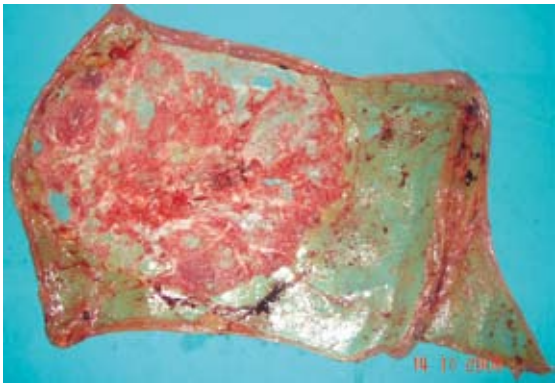


Figure 1 Preparation of chorioamniotic membrane from the placenta by removing the cotyledons under sterile conditions.



Figure 2 Suture of human chorioamniotic membrane to abdominal skin.



Figure 3 Appearance of the abdomen after membrane application.

reported that in cases where closure is inadequate, by using acellular dermis, patients can be sent home without a fascial defect[5]. However, there is no long-term follow-up of wide series on outcomes with this biological material.

In our patients, human chorioamniotic membrane obtained from elective cesarean sections was used instead of the plastic Bogota bag. It is a natural biological material, therefore, it is a more appropriate cover for erosion of the intestinal serosa. Moreover, chorioamniotic membrane accelerated adhesions among the loops and helped to stabilize the abdomen.

There are different indications for the use of placental amniotic membrane. It is used successfully to cover tendon defects, to prevent adhesions[6]. The membrane is also used for eye and skin diseases[7]. Previously, in order to prevent fistula of damaged bowel segments, fibrin glue, together with acellular dermal matrix, was used for covering the open abdomen. We have used human chorioamniotic membrane for the same purpose[8,9].

We use the chorioamniotic membrane because the amniotic membrane is very thin. The maternal surface is placed over the viscera. Another possibility is to place the membrane between the viscus and the peritoneum. Similar outcomes may be expected with this method and vacuum techniques. In the latter, the deepest part of the vacuum system which is plastic cover is placed between the peritoneum and the viscus to prevent the parietal peritoneum from adhering to the visceral peritoneum. In our method, because the fetal surface is not sticky and smooth, it might prevent the viscus from adhering to the peritoneum. In the present case, because it was impossible to keep the viscus in the abdomen, we deliberately aimed for adhesion between the parietal and visceral peritoneum.

We conclude that human chorioamniotic membrane prepared under sterile conditions may be an alternative to conventional plastics bags in daily practice, for preventing serosal erosion and fistulas in patients with an open abdomen. The biological membrane is cheap, practical and is well suiting over viscera. It is sutured easily and does not make plies like the plastic material in use.

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EDITORIAL

Laparoscopic sleeve gastrectomy for morbid obesity

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Abstract

The incidence of obesity is steadily rising, and it has been estimated that 40% of the US population will be obese by the year 2025 if the current trend continues. In recent years there has been renewed interest in the surgical treatment of morbid obesity in concomitance with the epidemic of obesity. Bariatric surgery proved effective in providing weight loss of large magnitude, correction of comorbidities and excellent short-term and long-term outcomes, decreasing overall mortality and providing a marked survival advantage. The Laparoscopic Sleeve Gastrectomy (LSG) has increased in popularity and is currently very "trendy" among laparoscopic surgeons involved in bariatric surgery. As LSG proved to be effective in achieving considerable weight loss in the short-term, it has been proposed by some as a sole bariatric procedure. This editorial focuses on the particular advantages of LSG in the treatment of morbid obesity.

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Key words: Obesity; Bbariatric surgery; Sleeve gastrectomy; Ghrelin; Weight loss

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INTRODUCTION

The incidence of obesity is steadily rising, and it has been estimated that 40% of the US population will be obese by the year 2025 if the current trend continues[1]. An alarming indicator of the health crisis related to the epidemic of obesity is that 15% of children and adolescents are obese (body mass index BMI > 95th percentile on the Centers for Disease Controls and Prevention standard charts), and over 20% are at risk (BMI > 85 percentile)[2]. In recent years there has been renewed interest in the surgical treatment of morbid obesity in concomitance with the epidemic of obesity, and application of the laparoscopic techniques to the field of bariatric surgery as well. Bariatric surgery proved effective in providing weight loss of large magnitude, correction of comorbidities and excellent short-term and long-term outcomes[3-5], decreasing overall mortality and providing a marked survival advantage[6,7]. The number of laparoscopic Roux-en-Y Gastric ByPass (LRYGBP) procedures have continued to increase in the last few years. This has contributed to the profound enthusiasm of the surgical community for bariatric surgery. In this context, new bariatric procedures can be rapidly accepted by the surgical community, as has already happened in the case of laparoscopic adjustable gastric banding (LAGB). The latter is a paradigm of the "surgical enthusiasm" for a bariatric procedure. LAGB has rapidly reached wide diffusion, giving the label of bariatric surgeon to any laparoscopic surgeon able to place a band around the stomach of an obese patient. However, with time this procedure proved not to be the panacea that one would have thought it to be after its introduction. Due to a high rate of long-term failure, the literature indicates fewer LAGB procedures and more Roux-en-Y gastric bypass (RYGBP) procedures are being performed, and surgeons are choosing laparoscopic RYGBP[8-10] The Laparoscopic Sleeve Gastrectomy (LSG) is being performed more frequently and is currently very "trendy" among laparoscopic surgeons involved in bariatric surgery. LSG is not a new operation as it is the restrictive part of a more complex malabsorpitive bariatric procedure; i.e., the biliopancreatic diversion with duodenal switch (BPD-DS)[11,12]. Gagner and coworkers adapted the BDP-DS to the laparoscopic approach[13], and then introduced the concept of the staged approach to very obese patients

with the LSG being the first step[14]. As LSG proved to be effective in the short-term in achieving considerable weight loss, it has been proposed by some to be used solely as a bariatric procedure.

SURGICAL TECHNIQUE

LSG involves a longitudinal resection of the stomach on the greater curvature from the antrum starting opposite of the nerve of Latarjet up to the angle of His. The first step of the procedure is the division of the vascular supply of the greater curvature of the stomach, which is achieved with the section of the gastro-colic and gastro-splenic ligaments close to the stomach. The greater curvature must be completely freed up to the left crus of the diaphragm to completely resect the gastric fundus that harbours the ghrelin secreting cells of the stomach. The second step of the procedure is the longitudinal gastrectomy that "sleeves" the stomach to reduce it to a narrow tube. A naso-gastric tube is used to obtain a precise calibration and to avoid stenosis of the gastric plasty. There is no consensus as to where to start the gastrectomy, and which calibre of the naso-gastric tube to use. Gagner recommended starting the gastrectomy 10 cm proximal to the pylorus[14]. Baltazar recommended starting 4 cm from the pylorus in the case of DS, and only 2 cm from it if the LSG is intended as the sole bariatric procedure[15]. Different calibres of naso-gastric tubes have been reported by different authors from 32 Fr[15] to 60 Fr[14] The rational for starting closer to the pylorus and using a small calibre bougie to fashion the gastric tube is to increase the restrictive character of the procedure. The final volume of the gastric tube has been reported to be as small as 60 mL[15] and as large as 200 mL[13,14]. The staple line can then be reinforced with sutures or Seamguard to reduce the rate of staple line leak or bleeding[16] (Figure 1).

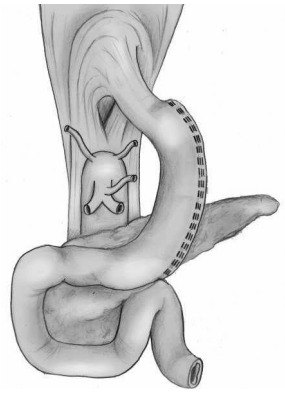


Figure 1 Laparoscopic sleeve gastrectomy.

PHYSIOLOGY OF LSG

One of the mechanisms involved in weight loss observed after the LSG is the dramatic reduction of the capacity of the stomach. The concept of restriction has been widely used in bariatric surgery in vertical banded gastroplasty (VBG) and LAGB. The distension of the small gastric pouch in the LAGB procedure or VBG is supposed to account for the feeling of early fullness, enhanced satiety and decreased hunger experienced by a patient after the ingestion of small quantities of food. The fact that the use of small calibre nasal-gastric tubes, as small as 30 Fr for LSG as proposed by Baltazar, provides faster and increased weight loss is in favour of the role of mechanical restriction as an important determinant in terms of weight loss that can be obtained with LSG[15].

The hormonal modifications induced by LSG differ from those found after a purely restrictive procedure as LASGB. Ghrelin, a peptide hormone mainly produced in the fundus of the stomach, is supposed to be involved in the mechanisms regulating hunger and has been a focus of interest in a growing number of recently published papers[17]. Ghrelin is secreted by the endocrine cells of the stomach (X/A-like cells), which reside in the oxyntic glands of the gastric fundus[18-20]. Gastric ghrelin producing cells are in contact with the basolateral membrane adjacent to the blood-stream and most of them do not come in contact with gastric content[18-21]. The gastric fundus contains 10 to 20 times more ghrelin per 1 × *g* of tissue than the duodenum[18-22] with diminishing concentrations being found in the jejunum and ileum[18,19,23]. Ghrelin has been also found in different additional tissues in very low concentrations such as the lungs, kidney, pancreatic islets, gonads, adrenal cortex, placenta and others[24]. Ghrelin regulates the secretion of growth hormone release and is a potent orexigenic (appetite-stimulating) peptide. This last effect of ghrelin is mediated by the activation of ghrelin receptors in the hypothalamus/pituitary area[25].

There is some evidence that body weight is the major determinant in the long-term regulation of the plasma concentration of ghrelin[26,27]. Accordingly, it has been shown that plasma concentration of ghrelin is increased in the case of negative energy balance situations such as low-calorie diets[26,28], chronic exercise[28,29], cancer anorexia[28], and anorexia nervosa[29], and are decreased in positive balance situations such as weight regain after overfeeding, or during the weight recovery phase of anorexia nervosa[30] or in obese patients[31]. However as no putative mechanisms have been elucidated so far to explicate the changes in the plasma levels of ghrelin in the long term, further studies are necessary to define this issue.

On the other hand, plasma concentration of ghrelin is acutely regulated by the intake of food with a meal to meal variation[31,32]. Plasma ghrelin concentration rises just before the onset of meal and declines thereafter suggesting that ghrelin could play a role in the signal of meal initiation[18,23,33,34], acting as the counterpart of the several meal-related satiation factors such as cholecystokinin, peptide -YY, glucagons-like peptide 1. Although the mechanisms responsible for the postprandial suppression of ghrelin are not known there is evidence that neither gastric nutrients nor gastric distension affect the plasma level of ghrelin[35]. Different studies investigated the plasma levels of ghrelin after different bariatric procedures, such as LASGB, LRYGBP and BPD[17]. Unfortunately, the results of these studies are difficult to compare because the patients studied were in different states of energy balance, the techniques of sampling were different, and either the fasting or postprandial ghrelin levels were reported[17]. Nevertheless, there is overall evidence that LASGB is

associated with significantly increased total plasma levels of ghrelin as opposed to LRYGBP, which is followed by a decrease of ghrelin[36-41].

As LASGB preserves the whole stomach, the gastric fundus, which is the major ghrelin producing tissue, is not excluded form contact with food. Ghrelin levels are increased with diet induced weight loss. Interestingly RYGBP has been shown to be associated with low levels of circulating ghrelin that persist at 1 and 3 years follow-up[42,43]. On the contrary, LASGB, VBG[44], and antireflux surgical procedures[37,45-48] are not associated with a decline of the circulating levels of ghrelin[42]. The most important difference between these procedures and LRYGBP is the contact of food with the gastric fundus of the stomach. The mechanism hypothesized to explain why ghrelin is down-regulated after LRYGBP is the override stimulation theory as described by Cummings. The empty stomach that is acutely associated with increased levels of circulating ghrelin would result in a continuous stimulatory signal that ultimately suppresses ghrelin production[23-26]. However, whether weight loss after LRYGBP is directly related to the changes in plasma levels of ghrelin is still a matter of debate[49].

Holdstock *et al*[50] found increased levels of ghrelin 12 mo after GBP and argued that weight loss is not directly related to plasma concentration of ghrelin. Faraj *et al*[51] proposed that the plasma levels of ghrelin are related to the state of energy balance. During the period of weight loss after GBP, ghrelin is secreted by sources other than the stomach because of the negative energy balance resulting in plasma levels higher than controls. Once patients have completed their weight loss phase and have reached a state of energy balance, plasma levels of ghrelin are comparable to those of controls despite massive weight loss The disparate results reported in the literature on the mechanism involved in weight loss after LRYGBP imply that the relationship between the mechanical effects of surgery and neuroendocrine response is complex and far from being clearly elucidated.

Lager *et al*[52] reported significantly reduced levels of ghrelin in 10 obese patients undergoing LSG at 1 and 6 mo after surgery. Interestingly, these patients were prospectively compared with 10 patients undergoing a LASGB procedure that resulted in significantly increased levels of ghrelin at 1 and 6 mo. This was paralleled by a superior weight loss in the group of patients undergoing LSG, which might be related to the low levels of circulating ghrelin with no compensatory hunger. In the case of BPD, the circulating levels of ghrelin are differently affected, depending on the type of gastrectomy that is associated with the BPD. In the case of BDP, according to Scopinaro, the gastric fundus is preserved and ghrelin levels are not reduced as reported by Adami *et al*[47]. On the contrary, when the BPD is associated with the pylorus preserving sleeve gastrectomy and duodenal switch (BPD-DS), the circulating levels of ghrelin are markedly suppressed[53].

Ghrelin seems to play a key role in the complex energy balance loop and it is logical to hypothesize that its changes are involved in the neurophysiologic mechanisms responsible for the changes in appetite observed after bariatric surgery. At the moment, the short term results of LSG are more similar to those observed after LRGBP than other purely restrictive procedures. Whether the observed change in circulating ghrelin level is a predictor of the long-term success of a given bariatric procedure, such as LSG, is not yet clear.

PRO AND CONS OF LSG

The seducing potential of LSG relies in the fact that this operation is a straightforward procedure that can be generally completed laparoscopically, even in the case of an extremely obese patient. It does not involve any digestive anastomosis, no mesenteric defects are created eliminating the risk of internal hernia[54], no foreign material is used as in the case of gastric banding, the whole digestive tract remains accessible to endoscopy, it is not associated with Dumping syndrome, the risk of peptic ulcer is low and the absorption of nutrients, vitamins, minerals and drugs is not altered.

However, some points need to be underlined at least for those who are too enthusiastic about LSG. First, although LSG is generally an easy procedure, it may become a true nightmare in the very obese patient, especially in men with a huge left liver lobe, and the long staple line may leak postoperatively. Secondly, in face of the eventual regain of lost weight or insufficient weight loss, the surgeon must be ready to do a second procedure that must be part of his technical armamentarium. The last problem is represented by the choice of the second procedure, which is traditionally the BPD-DS in the case of patients with a BMI > 50. Other teams propose the LRYGBP but it is still not clear whether the latter can provide further weight loss in the case of patients with an initial BMI > 50. The Scopinaro procedure or the VVLRYGBP are other alternatives, and the stomach might be re-sleeved in case of gastric tube dilation.

RESULTS OF LSG

Only a few studies on sleeve gastrectomy for morbid obesity have been published so far. By performing a search in PubMed MEDLINE (National Library of Medicine) for English-language articles published until March 2007 using the key words *laparoscopic, sleeve gastrectomy*, and considering only series reporting at least 30 patients, a total of 6 papers reporting a total of 328 cases was obtained[15,55-59]. Table 1 summarizes relevant data collected from these articles.

Mean initial BMI ranged from 37.2 kg/m^2[57,58] to 65.4 kg/m^2[56] with a case of a patient with an extreme BMI at 91 kg/m^2[56]. These data suggest that very high and even extreme BMI does not represent a contraindication to LSG. In fact, the two staged approach is becoming the rule in the case of super-super obese patients (BMI > 60 kg/m^2) in which more complex procedures such as RYGBP or biliopancreatic diversion are very difficult to perform. The rational of the staged approach to super and super-super obese patients is to achieve a substantial weight loss with the consequent amelioration of obesity-related comorbidities with a simple procedure such as LSG, thus allowing the second procedure in patients with lower operative risks. Of the 328 cases, data on gender

Table 1 Studies of Sleeve Laparoscopic Sleeve Gastrectomy including at least 30 patients

Study type	Sample size	Sex	Age(yr)	Mean follow up (mo)	Death (%)	Complications	Mean operative time (min)	Initial BMI	EWL (%)	Reference
Retrospective	31	NA	NA	3-27	3.2	n.1 bleeding (requiring laparoscopic reoperation)		Gr 1: > 61 Gr 2: > 40 Gr 3: 25-30 Gr 4: NA	NA	15
Retrospective	60	8 M	30 (16-62)	12	1.6	1 leak 1 delayed bleeding 1 atelectasis 1postoperative vomiting	70 (45-100)	37.2 (30-56.1)	NA	57
Retrospective	126	59 M	49.5 (20-74)	12	0.8	5 strictures 2 leaks 2 pulmonary embolus 5respiratory insufficiencies 4 renal failures	143 (90-120)	65.4 (45-91)	45 (for the 54 pts who underwent the phase Ⅱ operation LRYGBP)	56
Prospective	41	13 M	44.6 ± 9.7	22.2	0	1 leak 1 transient renal failure bleeding	111 ± 31	57.3 ± 6.5	NA (but average BMI after 1 yr was 40.8 ± 8.5)	55
Randomized prospective (LSG vs gastric banding)	40	9 M	40 (22-65)	36	0	1 bleeding 1 gastric ischemia	NA	39 (30-53)	66	58
Retrospective	30	7 M	40 (17-69)	6	0	3 dehydrations 1 leak	80 (65-130)	41.4 (33-59)	52.8	59

NA: Non Applicable; M: Men; EWL: Excess Weight Loss; min: Minutes; pts: Patients; ref: Reference.

were available for 297 cases, for a total of 96 males (32%) and 201 females (68%) undergoing the procedure. In fact, beside high BMI, male gender is another important risk factor for postoperative morbidity and mortality and is associated with technical difficulties at the time of surgery. Men, in fact, often have an android body habitus whereby excess body fat is localized in the peritoneal cavity and the left lobe of the liver is often enlarged rendering the access to the stomach and hiatal region difficult[55]. Thus, the relatively high percentage of male patients undergoing SG in the literature reflects the choice of the bariatric surgeon in favour of SG, which is less challenging than other procedures requiring larger dissections and intestinal anastomoses.

In 3 of the 6 studies analyzed (total of 130 patients)[57-59], LSG was performed as the sole procedure; i.e. not followed by other types of bariatric operations. However, one of these patients[57] underwent a laparoscopic gastric bypass one year after LSG because he failed to achieve a satisfactory weight loss. In the last 3 studies, enrolling a total of 198 patients[15,55,56], LSG was intended as the first step of a combined approach to achieve an initial weight loss in "difficult" patients with the second step being a LRYGBP or DS. Among the 198 patients undergoing the LSG as the first step of the combined approach strategy, 51 patients finally underwent the second procedure: 36 had a LRYGBP and 15 a DS. Interestingly, in the 3 studies in which LSG was performed as a first step procedure, only a part of the initially programmed second procedures were finally performed. It can be speculated that at least in some of the cases the weight loss achieved after LSG alone was satisfying[55,56,59].

Mean operative time was clearly reported in 4 of the 6 studies[55-57,59], ranging from 70 min[57] to 143 min[56]. As expected, the operative time is longer in patients with high BMI.

Unfortunately complete data about excess weight loss are reported in 3 of the 6 studies[57-59]. This is a main concern as the primary outcome of bariatric surgery is excess weight loss, and a given procedure cannot be evaluated correctly if the functional results are not reported. Mean excess weight loss ranged from 52.8% (follow up 6 mo)[59] to 83.3% (follow up 12 mo).

Three postoperative deaths were recorded for 328 patients (death rate of 0.9%) and a total of 34 major and minor complications were reported (morbidity 10.3%). Fifteen of these were considered minor complications: 5 transient renal failures not requiring dialysis, 5 patients requiring ventilatory support for > 24 h, 1 pulmonary atelectasis, 1 case of prolonged postoperative vomiting treated conservatively and spontaneously resolved, 3 dehydrations. By excluding minor complications, a total of 19 major complications occurred (major complication rate 5.8%). Major complications managed conservatively included 4 cases of staple-line leaks, 5 strictures of the gastric plasty, 2 cases of bleeding and 2 cases of pulmonary embolus[55-57]. Six patients with major postoperative complications required re-operation. Re-operations were performed by laparoscopy in 4 cases of intraperitoneal bleeding[12,49,51,52]. One case of staple-line leak[59] and a case of gastric ischemia required re-operation by laparotomy[58].

Bariatric surgery is associated with a mortality rate of 0.1-2.0 in large studies[60-64]. In a recent meta-analysis by Buchwald *et al*[65], postoperative mortality was 0.5% for RYGBP, 0.1% for gastric banding, and 1.1% for malabsorpitive procedures. In one of the largest prospective studies[66], the SOS trial, postoperative complications occurred in 13% of patients, including

bleeding in 0.5%, embolism or thrombosis in 0.8%, wound complications 1.8%, and pulmonary complications in 6.1%. In a recent review of the literature, Baker *et al*[67] reported an average rate of leak after LRYGBP of 2.77% (range 0.3% to 8.3%).

The evolution of obesity related comorbidities after SG has not been clearly reported. However, SG should be effective in improving comorbidities, such as non alcoholic fatty liver disease/nonalcoholic steatohepatitis (NAFLD/NASH)[68], as with any other procedure that induces weight loss because the improvement of comorbidities is directly dependent on weight loss. Overall these results suggest that LSG is safe and effective in the short term to achieve a substantial weight loss. There is no evidence so far that weight loss obtained with LSG can be maintained in the long term.

WEIGHT REGAIN AFTER LSG INDUCED INITIAL WEIGHT LOSS

In other words, can LSG be proposed as a sole bariatric procedure? When the LSG is done as the first step of a staged approach in very obese patients, the second procedure is generally done when the weight loss reaches a plateau, in the case of weigh regain or simply when the patient can undergo more safely the second step regardless of weight loss stabilization or regain. The possible mechanisms that account for the limited weight loss or weight regain include a technical problem, such as an incomplete resection of the fundus of the stomach where the ghrelin producing cells are located. This may occur in patients with hiatal hernia, which is often associated with excessive body weight[69]. Gastric dilatation has been evocated as being responsible for poor weight loss after LSG with or without DS[70]. An excessive large pouch may be the result of an excessively large pouch created at the initial operation due to a too large calibration tube, or because of inappropriate surgical technique such as missed posterior gastric folds. Excessive pressure against the pouch wall by large meals, repeated vomiting or distal obstruction may also account for pouch dilation[71]. Other factors may be claimed as responsible for poor weight loss or weight regain after LSG, such as the spontaneous dilation of the gastric tube over time, the switch to a high caloric, soft, liquid diet, and, last but not least, LSG may have a limited potential for weight loss as do most restrictive procedures.

The question whether the LSG may work as a sole bariatric procedure in the long term cannot be answered yet. For this reason LSG should be proposed as the first step of a staged approach in patients for whom a BPD-DS or LRYGBP seems too hazardous because of very high BMI (super obesity-BMI > 50 or super-super obesity-BMI > 60), and/or associated diseases whether related or not to obesity. The decision to do a LSG may also be made during surgery in the case of intraoperative findings, such as liver cirrhosis, adhesions, too thick mesenterica contraindicating a more complex procedure. Then, the second step may be deferred until deemed necessary because of insufficient weight loss or weight regain. On the other hand, the LSG should not be proposed to patients with a BMI < 50 until evidence is provided for its effectiveness in achieving weight loss in the long term compared to the results obtained with RYGBP, which remains the gold standard of bariatric procedures at the moment. Exceptions might warrant a pure restrictive procedure, such as for patients with inflammatory bowel disease for whom the intestinal bypass is not advised, and transplanted patients for whom the intestinal bypass may interfere with the absorption of immunosuppressive drugs.

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OBSERVER

Refractory celiac disease and sprue-like intestinal disease

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Abstract

Celiac disease is a gluten-dependent small intestinal mucosal disorder that causes malabsorption, often with diarrhea and weight loss. Diagnosis is based on detection of typical biopsy changes in the proximal small bowel, followed by evidence for an unequivocal response to a gluten-free diet. Refractoriness in celiac disease may be due to poor diet compliance, sometimes intentional, or consumption of ubiquitous sources of gluten. Alternatively, the original diagnosis may not be correct (eg., duodenal Crohn's disease), or a second cause for symptoms may be present (eg., collagenous colitis, functional bowel disorder). In some with recurrent symptoms, a complication may be present (eg., collagenous sprue, small bowel carcinoma, lymphoma). In some, a response to a gluten-free diet can not be unequivocally defined, and more precise historical terms have been used including "sprue-like intestinal disease" or "unclassified sprue". Although a "wastebasket diagnosis", these likely represent a heterogeneous group, and some, but not all, may develop lymphoma. Precise definition will be critical in the future as an array of new treatments, including biological agents, may emerge.

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Key words: Refractory celiac disease; Refractory sprue; Unclassified sprue; Celiac disease; Intestinal lymphoma; T-cell enteropathy

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INTRODUCTION

Celiac disease is a gluten-dependent malabsorption disorder that involves the small bowel and can cause diarrhea and weight loss. Diagnosis is based on two criteria: first, proximal small intestinal mucosal biopsies that show typical biopsy abnormalities; and second, evidence for an unequivocal response to a gluten-free diet. Severe ("flat") or variably severe small bowel mucosal architectural abnormalities are present with crypt epithelial cell hyperplasia and villous atrophy. Lymphoid cell changes also occur, including intraepithelial lymphocytosis. Alterations are most severe in duodenum and proximal jejunum, and less severe, or absent, in ileum. Although characteristic, these findings are not specific or diagnostic alone as several disorders can produce similar, but not necessarily, identical histopathological changes (Table 1). Some serological assays (eg., tissue transglutaminase antibodies, or tTG) are useful screening tools in adults, but these alone do not permit a definitive diagnosis. The most critical step in the diagnosis of celiac disease is definition of a gluten-free diet response. Usually, with a gluten-free diet, diarrhea resolves and weight gain occurs. Histopathologic changes in the small bowel normalize, initially in distal intestinal sites of involvement, and later, sometimes only after prolonged periods (even months to years), in the proximal duodenum[1,2]. Recent studies have also shown that normalization of duodenal biopsies may take even longer in the elderly[3].

REFRACTORINESS IN CELIAC DISEASE

Recurrent symptoms may occur in established celiac disease (Table 2). Most often, this appears to be due to poor compliance with a strict gluten-free diet, although compliance is sometimes very difficult to define or fully ascertain. Intentional dietary indiscretion may be obvious, or, alternatively, there may be limited awareness of gluten-containing food sources. Gluten is so ubiquitous, known to be present in pill capsules and communion wafers, as well as in a host of processed food products. Gluten-free foods may be quite expensive, limited in their palatability, and, especially in some developing countries, difficult to obtain. Moreover, professionals, including physicians and dietitians, support groups and the internet, all represent potential sources of inaccurate information. Motivation to follow a strict diet may be limited if symptoms are minimal or absent when "cheating" occurs. Finally, social or peer pressure, especially during adolescence or early adulthood may also hinder efforts to maintain compliance.

In some with recurrent symptoms, however, other causes may be responsible. It is possible that the original

Table 1 Some causes of biopsy changes similar to celiac disease

Causes	Diseases
Sprue syndromes	Collagenous sprue
	Mesenteric lymph node cavitation syndrome
	Oats-induced villous atrophy
	Other protein injury (soy, milk)
	Unclassified or refractory sprue
Infectious causes	Infectious gastroenteritis
	Specific infections (eg., parasite: strongyloidiasis, Protozoan: giardia, mycobacteria)
	Tropical sprue
	Stasis syndrome (contaminated small bowel syndrome)
	Whipple's disease
Deficiency causes	Nutrients (zinc, vitamin B12, folic acid)
	Kwashiorkor
	Immunodeficiency syndromes
Others	Intestinal lymphangiectasia
	Crohn's disease (duodenum)
	Graft-versus-host disease
	Immunoproliferative disease (lymphoma)
	Macroglobulinemia
	Zollinger-Ellison syndrome
	Drug-induced small bowel injury (eg. NSAIDs)

diagnosis was incorrect, especially if there was only limited attention to defining the response to a gluten-free diet. And, there are many conditions that may cause a virtually identical histopathological small bowel lesion[4]. In addition, a second cause for symptoms may have developed. These may include other associated or linked disorders, like collagenous colitis[5] or even a functional bowel disorder. Alternatively, in those that appear to have "refractory" symptoms, a complication may have developed (eg., collagenous sprue, small intestinal carcinoma, lymphoma)[6]. In some with persistent symptoms and architecturally abnormal biopsies, clonal expansion of an aberrant intra-epithelial lymphocyte population has been reported (so-called type 2 disease versus type 1 disease with apparently normal intraepithelial lymphocyte phenotype). This condition has been labeled "cryptic T-cell lymphoma" as there appears to be a higher risk of overt T-cell lymphoma[2,7].

UNCLASSIFIED SPRUE OR "SPRUE-LIKE INTESTINAL DISEASE"

Sometimes, the small bowel biopsy changes do not appear to improve despite apparently good compliance on a gluten-free diet. Persistent symptoms and changes in the small bowel biopsies are present. Although a relatively treatment-resistant form of celiac disease could be present, possibly with a distal small bowel diet response, it is more likely that celiac disease is not present at all. Rather than labeling these patients with refractory celiac disease, more precise terms have been historically used including "sprue-like intestinal disease" or "unclassified sprue"[8].

In these, persistent symptoms and ongoing pathological changes are present despite a strict gluten-free diet. In some reports, persistent pathological abnormalities refer to results of repeated endoscopic biopsies from the proximal small bowel during an arbitrary time period of 6 mo to up to one year. Rarely, there is a rapidly progressing disease course that makes it difficult to show a convincing response to a gluten-free diet. While clinical and pathological changes are reminiscent of celiac disease in some of these patients, there is usually no convincing evidence that a gluten-free diet response ever occurred. "Sprue-like intestinal disease" or unclassified sprue is a "wastebasket" disease diagnosis that appears to represent a heterogeneous group of different disorders. Some eventually develop lymphoma, but some do not. A few are reported to have epithelial cell antibodies, specificially anti-enterocyte and anti-goblet cell antibodies. These have been reported in both children or adults with persistent diarrhea and severe biopsy changes, similar to untreated celiac disease. In these, no response to a gluten-free diet or any dietary exclusion occurs. Some believe that these represent a distinct autoimmune enteropathic disorder[10].

Table 2 Recurrent or refractory symptoms in celiac disease

Dietary non-compliance
Ubiquitous gluten source (eg., pill capsules)
Wrong initial diagnosis
Associated or second cause (eg., collagenous colitis)
Superimposed complication (eg., collagenous sprue, lymphoma)

FUTURE DEVELOPMENTS

Refractory disease represents a complex array of difficult clinical problems, in part, because definitions are evolving and there is no clear consensus for treatment, unless a lymphoma has already been detected. In case reports of refractory disease with clonally expanded T-cell populations, use of infliximab[11] and cladribine[12] were described, but progression to overt lymphoma occurred. In addition, use of autologous hematopoietic stem-cell transplantation has also been done, but with mixed results, especially if overt T-cell lymphoma is present[13-15]. Better refinements in the definition of refractory diseases are needed, possibly using genetic markers to identify risk[16]. These may eventually aid in treatment, especially with emergence of an array of new biological agents for treatment of immune-mediated and malignant disorders.

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REVIEW

The blind men 'see' the elephant-the many faces of fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a group of diseases with excess fat in liver in the absence of a poorly defined limit of alcohol consumption. Most common variety, a universal public health problem, is associated with insulin resistance caused by a host of genetic and epigenetic defects modulated by life style and environmental factors. In fact the term NAFLD is loose to incorporate so many etiologies except alcoholism and few other etiologies, presenting as fat in liver. However as a sign fatty liver is very important in predicting the risk of diabetes, cardiovascular disease, stroke, cirrhosis and cancer. Abnormal fat accumulation can result from several defects in nuclear receptors associated with lipid sensing, synthesis and oxidation like LXR, FXR, SREBP, ChREBP and PPAR; defects in the lipid influx-efflux channels, insulin signaling, proteins involved in fatty acid catabolism, defects in adipose tissue development and function, inappropriate nutrition and finally defects in neural regulatory mechanisms. The progress of the disease is determined by the basic defects which results in fat accumulation, an individual's immunological response to the accumulated fat and its derivatives and the oxidant stress response. Congregation of unrelated genetic defects under same diagnosis 'NAFLD' can result in inefficient patient management. Further studies are required to understand the molecular basis of fatty liver to enable a personalized management of diseases presenting as fatty liver in the absence of alcohol abuse.

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Key words: Nonalcoholic fatty liver disease; Insulin resistance; Lipid homeostasis; Primate evolution; Lipid channels; Lipoprotein; Adipokines; Nuclear receptors; Bile acid metabolism; Personalized medicine

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INTRODUCTION

"It was six men of Hindustan, To learning much inclined, Who went to see the Elephant, (Though all of them were blind), That each by observation, Might satisfy his mind. "God bless me! but the Elephant: Is very like a wall! Is very like a spear! Is very like a snake! Is very like a tree! Is very like a fan! Is very like a rope!" And so these men of Indostan, Disputed loud and long, Each in his own opinion, Exceeding stiff and strong, Though each was partly in the right, And all were in the wrong!"

-John Godfrey Saxe (1816-1887).

Although alcohol-induced liver steatosis was already described by Thomas Addison in 1845, it is appreciated only since 1962 that steatosis can also occur without the use of alcohol, so-called non-alcoholic steatosis[1]. The term nonalcoholic steatohepatitis (NASH) was coined in 1980 to describe "the pathological and clinical features of non-alcoholic disease of the liver associated with the pathological features most commonly seen in alcoholic liver disease itself"[2]. In fact, the finding that some obese individuals had a liver disease histologically indistinguishable from alcoholic liver disease itself had long been recognized.

The etiopathogenesis of nonalcoholic fatty liver disease (NAFLD) has been disputed loud and long (Figure 1). NAFLD is a vague term for a spectrum of diseases which differ not only in the presentation but also in the etiologies which warrants a personalized approach in the diagnosis and treatment of this condition. All possible etiologies and pathogenesis models have been assigned to it from mitochondrial, oxidant stress, hormonocentric to adipocentric models[3,4]. On the other hand the term NAFLD is loose to incorporate so many etiologies except alcoholism and few other etiologies, presenting as fat in liver. Interestingly despite the diversity in root cause and irrespective of the fact they fall inside or out side the conventional inclusion or exclusion criteria many of them share common pathways of disease progression evolution and termination. Here in this review, we once again review the 'elephant' and attempt to classify and generalize the disorder based on the etiology.

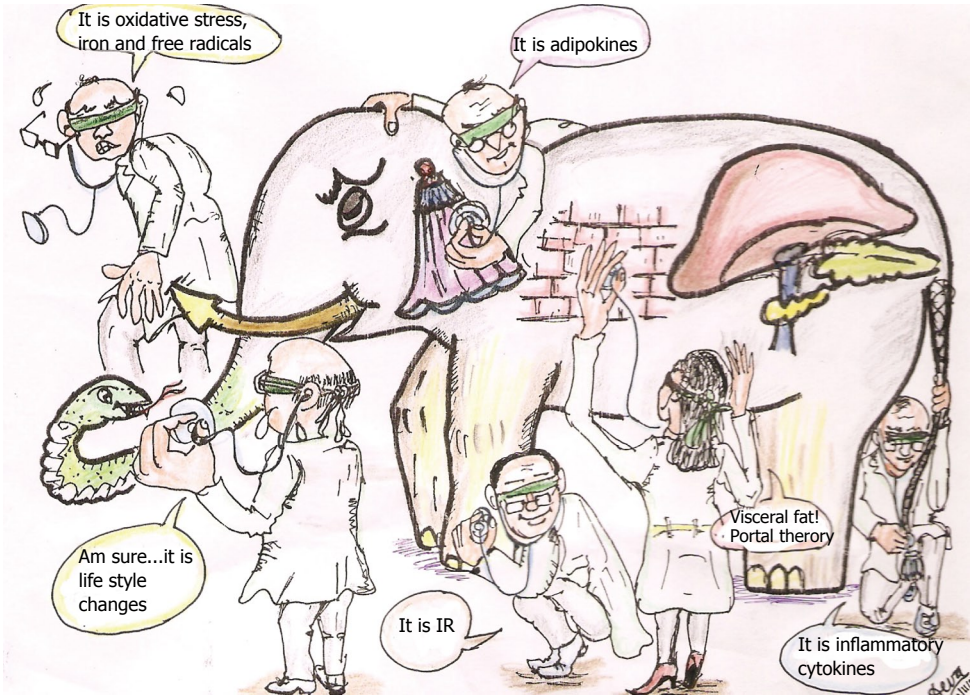


Figure 1 The pathogenesis of NAFLD has been disputed by many loud and long. The term "nonalcoholic fatty liver disease" is imprecisely defined; there is uncertainty regarding its pathogenesis. The elephant (NAFLD) has been interpreted by many and several possible etiologies and pathogenesis models have been assigned to it like 'insulin resistance', mitochondrial defects, adipokine imbalance, visceral fat, inflammatory cytokines and oxidant stress.

NAFLD: A nonspecific diagnosis and a misnomer?

A variety of genetic defects and pathogenic pathways can result in NAFLD. Fatty liver is a sign rather than a proper diagnosis. As a sign it is very important in predicting the possibility of diabetes, cardiovascular risk, stroke, cirrhosis and cancer. Symptoms, severity and prognosis for same amount of fat in liver differ very much from person to person and fat may be totally absent in late stages of the disease. In addition, there is no genetic or clinical marker specific for NAFLD. Opinion regarding the histopathological diagnosis and staging differ even among experts and there is a lack of reproducibility[5,6]. Furthermore, authors have differed on the level of alcohol consumption that can reliably distinguish between alcoholic steatohepatitis and NASH.

"Nothing makes sense in biology without the concept of evolution"

'Nothing in Biology Makes Sense Except in the Light of Evolution' is a 1973 essay by the evolutionary biologist Theodosius Dobzhansky, criticizing Young Earth creationism and espousing evolutionary creationism makes sense with 'NAFLD' as well.

The ability to obtain food and manage periods of starvation and the ability to fight infectious diseases were most critical in survival of primates and indeed their survival was threatened by starvation and infectious diseases than obesity and autoimmunity or cancer. So, primate evolution always favored "thrifty genotypes"[7] which were efficient in fat storage and burning. Similarly 'infection resistant' genotypes were favored by evolution. The cultural evolution was faster so that the 'genetic-evolution' lagged behind or became static. As we 'evolved' from hunter-gatherer life style to computer savvy-chair bound, calorie rich-fast food friendly and 'hygienic' life style our past started haunting and our 'vestigial genes' started their manifestation in the form of the pandemic called Metabolic Syndrome with associated insulin resistance (IR), NAFLD, chronic inflammatory disorders and cancer.

Evolutionary history of adipose tissue, liver and immunocytes adds more sense to this picture. It is interesting to note that all the above has same ancestor-the "fat body" which is the mammalian homologue of all the three tissues in insects[8,9]! Over expressing peroxisome proliferator-activated receptor gamma (PPAR-γ) a gene that is vital for differentiation and maturation of adipocyte genes in hepatocytes can induce adipocyte like features in hepatocytes. Similarly gene expression profile of macrophage is very similar to adipocyte[10-13]. Hence all the three systems are simultaneously affected in disorders like NAFLD (Figure 2). Indeed in fatty liver disease, hepatocytes become lipid-loaded and resemble adipocytes with the formation of large lipid droplets, expression of adipogenic and lipogenic genes such as adipose differentiation-related protein (ADRP), PPAR-γ and sterol regulatory element binding protein (SREBP)[3,9-13]. The interactions and involvement of the trio in development of fatty liver is discussed further in this article.

NAFLD AS THE LIVER MANIFESTATION OF A GENERALISED FAT STORAGE DISORDER

Logically if fat has to get accumulated there are four ways (Figure 3): (1) increased inflow (increased availability of lipids and lipid precursors); (2) decreased outflow/secretion; (3) increased synthesis; and (4) decreased oxidation.

Increased inflow

It has been observed that nearly 80% of people who undergo liver transplantation following cirrhosis from NASH get back the disease in few years[14]. This point

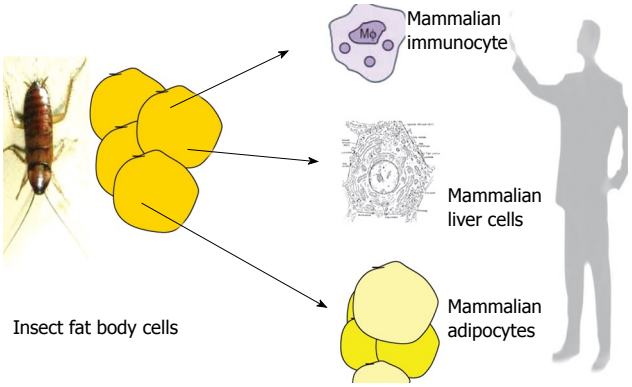


Figure 2 It is interesting to note that adipose tissue, liver and immunocytes has same ancestor-the "fat body" which is the mammalian homologue of all the three tissues in insects. Over expressing PPAR-γ in hepatocytes can induce adipocyte like features in hepatocytes. Similarly gene expression profile of macrophage is very similar to adipocytes. All the three cell systems are affected in disorders like NAFLD and obesity.

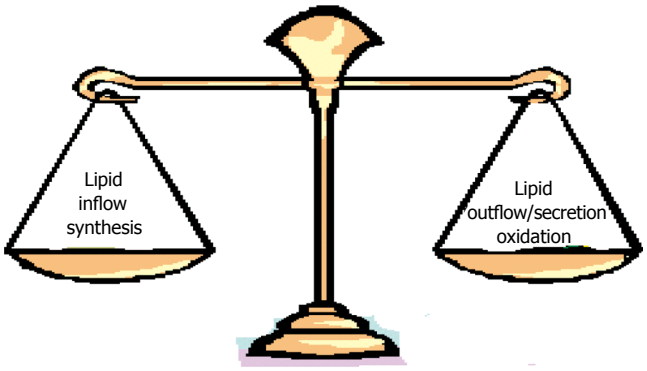


Figure 3 Fat gets accumulated in liver when there an imbalance between inflow (increased availability of lipids and lipid precursors) and outflow/secretion, synthesis and oxidation.

to the fact that at least in majority of the patients the cause lays outside the liver. Adipose tissue functions as an energy reserve; where energy can be stored in the most concentrated form viz. lipids safely unlike other tissues where it could cause 'lipid toxicity'[15]. This way it also acts as an energy sink in times of plenty. Soon after feeding the blood would be over loaded with energy rich compounds and the adipose tissue has an important role in clearing these compounds, especially lipids which are potentially harmful. However in chronic over nutrition adipocytes may become overloaded and may no longer be able to take up circulating lipids and glucose. Insulin is a stimulator of lipoprotein lipase the enzyme which mediates extraction of fatty acids from circulating lipoproteins which are the carriers of lipids as triglycerides (TG). Through the tissue-specific action of lipoprotein lipase, the TG-derived free fatty acids (FFA) are taken-up by peripheral tissues. Similarly the type-4 glucose transporters on adipocyte surface are also insulin dependent[16]. Probably the over loaded adipocytes may adopt insulin resistance (IR) as a strategy to save them selves from further overloading, damage and cell death (apoptosis?). As soon as the energy sinking capacity of the adipose tissue is exceeded the energy rich substrates starts 'over flowing' to other tissues like liver and muscle. This could be only one but the commonest mechanism of fatty liver. Nearly 60% of TGs deposited in liver in NAFLD comes from circulating nonesterified fatty acids[17]. The TGs contained within adipose tissue are continuously being hydrolyzed into fatty acids and glycerol by the enzyme hormone-sensitive lipase (HSL). Insulin resistance impairs uptake of glucose from blood into skeletal muscle and adipose tissue; serum non-esterified fatty acid (NEFA) levels may also be elevated due to the failure of insulin to suppress HSL mediated lipolysis[16].

However (1) other mechanisms which induces IR in adipose tissue or defects in the transporters of lipids into adipose tissue which limits influx of lipids, (2) defects in the control mechanisms involved in the release of lipids from adipocytes, (3) defects in normal growth and proliferation of adipose tissue, could result in plasma lipid pooling and ectopic fat deposition such as fatty liver. As in the case of adipocytes hepatic fat over load may lead to hepatic insulin resistance (HIR)[18]. Therefore it should be possible to reverse fatty liver by 'unloading' the extra fat. This is indeed true. For example surgical obesity management techniques like gastric banding based on restriction of the gastric reservoir produces early satiety and reducing oral intake induces significant weight loss by unloading of fat reserves reverses fatty liver. Exercise and diet restriction and consequent fat reduction still remains the mainstream therapy in NAFLD[19,20]. Interestingly removal of excess fat by liposuction doesnot improve insulin sensitivity[21]. This is possibly because as adipocytes are removed the buffering action provided by them on circulating energy rich substrates is decreased.

A small part of the fatty acids released by HSL as described before are re-esterified within adipocytes while most overflow into the blood which exceeds the normal oxidative needs of the body. Liver is another immediate buffer that has been shown to have a high capacity for accumulating fat and redirecting or oxidizing it later depending on the energy homeostatic signals. Within the liver, these fatty acids are either oxidized or re-esterified into TGs and secreted into the blood bound to VLDL[16,18]. The fatty acids re-esterified by the liver into TG are almost exclusively from adipose tissue lypolysis[22].

Gluconeogenesis in liver needs supply of fatty acids and glycerol released from adipocytes. Glycerol contributes 90% of the substrate for hepatic gluconeogenesis in prolonged fasting mice. In man about 20% of hepatic gluconeogenesis is mediated by glycerol. Gluconeogenesis needs a coordinated efflux of glycerol from adipocytes and influx into hepatocytes through adipocyte specific glycerol channel, designated aquaporin adipose (AQPap/7) and a liver-specific aquaglyceroporin was also identified and named AQP9[23]. Discord between efflux and influx channels possibly play an important role in the pathogenesis of NAFLD and diabetes mellitus.

Over flow of fat to liver could be due to increased dietary intake. Even after a short term fat feeding liver fat increases three fold without increase in visceral or skeletal muscle fat[18]. Indeed the adipose tissue fat is an indicator of liver fat. The intrahepatic lipids increase by 22% for

any 1% increase in total adipose tissue, by 21% for any 1% increase in subcutaneous adipose tissue and by 104% for 1% increase in intra-abdominal adipose tissue[24]. Thus liver bears the brunt as soon as adipose tissue buffering reaches its limit.

Zucker rats (*fa/fa*) have inactivating mutation in the leptin receptor and hence obese and develops fatty liver. Liver specific correction of leptin receptor deficiency results in reduced TG accumulation in the liver but not in other non-adipose tissues. This could be an example of adipokine mediated communication between adipose tissue and liver. Leptin in conditions of 'calorie excess' signals liver to increase lipid oxidation and to down regulate lipid synthesis and thus protect it and other organs from steatosis[25]. Ob/ob mice with mutations in Scd1 had histologically normal livers with significantly reduced triglyceride storage and VLDL production. Down regulation of SCD1 is an important component of leptin's metabolic actions[26].

As the buffering capacity of liver also exceeds its limit lipid starts getting accumulated in cardiovascular system and other organs. The macrophages associated with blood vessels, the evolutionary kin of adipocytes and hepatocytes, will try to "buffer" the excess lipids by phagocytosis and later oxidize them partly to form toxic free radicals and immunogenic compounds. They become 'foamy cells' as their capacity to accumulate fat reaches the limit[27]. This eventually leads to "fibrosis" of vessel wall (atherosclerosis is homologous to cirrhosis of liver) and an increase in CVS related mortality. Indeed some studies published recently find the seeds of CVS mortality in NAFLD[28-30] (Figure 4).

Defects in fatty acid transporters in adipocytes also could result in hypertriglyceridemia and subsequent increased inflow of FA to liver. For example

CD36-/-mice lacking the fatty acid transporter that is normally present in muscle and adipose tissue showed increased hepatic TG content and a decreased sensitivity of hepatic glucose production to insulin[31].

There are multiple genetic defects which culminate in adipose tissue deficiency and adipose tissue deficiency invariably results in fatty liver. Lean transgenic mice that express ZIP/F-1 protein in adipose tissue, which blocks the function of several classes of transcription factors, are insulin resistant and developed fatty liver. Interestingly in these mice upon transplantation of fat tissue insulin resistance as well as the fatty liver disappeared[32]. PPAR-γ mutant mice develop IR and fatty liver in conjunction with the terminal atrophy of adipose tissue[33]. Sterol regulatory element-binding proteins (SREBPs) are a family of transcription factors that activate the entire program of cholesterol and fatty acid synthesis. The transgenic mice that overexpress nuclear SREBP-1c (aP2-SREBP-1c) exclusively in adipose tissue have very little adipose tissue, apparently as a result of disturbed adipocyte differentiation. They develop severe hyperglycemia, hyperinsulinemia, and lipid accumulation in liver[34,35]. The fatty liver dystrophy (*fld*) mutant (lipin-deficient) mice are characterized by neonatal fatty liver and hypertriglyceridemia that resolve at weaning, glucose intolerance and increased susceptibility to atherosclerosis and neuropathy. Adult *fld/fld* mice exhibits 80% reduction in mass both white and brown fat pads compared with wild-type controls, and consist of immature adipocytes[36]. Human examples of adipose tissue deficiency and consequent fatty liver is described else where in this paper.

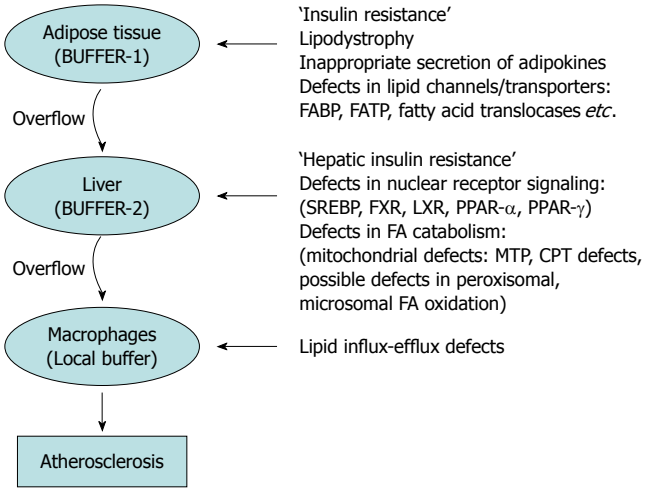


Figure 4 Adipose tissue functions as an energy reserve; where energy can be stored in the most concentrated form viz. lipids safely. In other tissues lipid accumulation could cause 'lipid toxicity'. Adipose tissue 'buffers' the lipids and energy rich compounds which are pumped into the blood stream soon after meals. However when excess calories overload adipose tissue the buffering action ceases, resulting in overflow of lipids to liver which is the next buffer. But as soon as liver also is overloaded, lipids overflow and gets accumulated in various other organs. In blood vessel walls macrophages takes up the excess lipids to form foam cells initiating a cascade which results in fibrosis of vessel walls.

Increased intake

The lipid intake is determined by lipoproteins which are carriers of lipids to liver predominantly from adipose tissue and digestive tract through the circulatory system. The lipids are further transported into hepatocytes by various 'lipid transporters' which are membrane bound channels and cellular lipid binding proteins like fatty acid transport proteins (FATP), fatty acid translocase (FAT/CD36), fatty acid binding proteins (FABP), caveolin-1 *etc*. Tissue accumulation of FA requires intracellular trapping involving association between many membrane' intracellular FA binding proteins.

FATP is highly expressed in hepatocytes and adipocytes that reveal high-level FA uptake for both metabolism and storage. FATP1 is found in adipose tissue and in the heart. FATP2 and FATP5 are expressed in the liver, while FATP4 is expressed in the intestine[37]. Overexpression of FATP5 in cultured cells has been shown to increase FFA uptake while knock out of FATP-5, results in decreased accumulation of fat in liver and decreased production of conjugated bile acids[38-40]. A recent study found an upregulation of FABP4 and FABP5 in NAFLD independent of obesity[13]. Thus FATP5 is an important membrane protein involved in fatty acid accumulation by the liver. In mice, which are FAT/CD36-deficient, the flux of fatty acids toward the liver is increased, precipitating steatosis, but there without any evidence of an increase in hepatic VLDL production[41].

The fatty acid taken up by liver is oxidized and excess is esterified and accumulated or secreted. Esterification is most

efficient with mono-unsaturated lipids as monounsaturated fatty acyl-CoAs are the preferred substrates for the synthesis of triacylglycerol (TAG) in the endoplasmic reticulum (ER)[42]. This is possibly one reason why loss of members of desaturation enzyme family, stearoyl-CoA desaturase which catalyses the rate-limiting step in the synthesis of monounsaturated fatty acids, particularly oleate (18:1) and palmitoleate (16:1), protects mice against fatty liver in mice[43].

The decreased outflow

The increased hepatic uptake and biosynthesis of FAs are compensated through increased removal of lipids from the liver. In this process, VLDL plays a central role. The principal apoprotein for this particle is apoB100, but apoE and apoC-Ⅰ, C-Ⅱ, and C-Ⅲ are incorporated as well[44]. Lipid homeostasis in mammalian cells is regulated by a family of membrane-bound transcription factors designated sterol regulatory element-binding proteins (SREBPs). In the liver, three SREBPs regulate the production of lipids for export into the plasma as lipoproteins and into the bile as micelles. nSREBP-1a transgenic mice develop a massive fatty liver engorged with both cholesterol and triglycerides[45].

The bile acid receptor farnesoid X receptor (FXR; NR1H4) is a central regulator of bile acid and lipid metabolism. FXR protects the liver from the deleterious effect of bile acid overloading by inhibiting their biosynthesis and stimulating their excretion. FXR regulates the expression of several apolipoproteins involved in the transport and metabolism of lipids[46,47] (Figure 5).

Bile acid reduces the secretion of VLDL by repressing microsomal triglyceride transfer protein (MTTP) which mediates lipidation of apoB100 to form VLDL[48]. This effect is possibly mediated through FXR. Pharmacologic agents that induce hepatic steatosis like amiodarone, tetracycline, pirprofen, tianepine inhibited MTTP activity[49]. However it is still unclear whether FXR mediated decrease in triglyceride rich VLDL secretion and consequent decrease in lipid out flow from liver is important in the pathogenesis of fatty liver.

Abetalipoproteinemia, a genetic disease which is associated with fatty liver is caused by mutations in the MTTP gene resulting in blockage of VLDL assembly and secretion[48]. MTTP-493 G/T polymorphism may influence NASH by modulating postprandial lipemia and lipoprotein metabolism; homozygous GG carriers have a more atherogenic postprandial lipid profile than the other genotypes, independently of adipokines and insulin resistance[50]. Fatty acid level is sensed in liver by PPAR-α and genes which promote lipid secretion like FABP, MTTP and apoB100 are upregulated[51-53]. Indeed this could be one mechanism by which fibrates which are PPAR-α agonists used in the treatment of NAFLD exerts its effect. Since lipid synthesis should go hand in hand with lipid secretion; as lipogenesis is increased by activation of the liver X receptor, hepatic VLDL production is also increased. Selective modulators of nuclear receptors involved in lipid homeostasis could thus revolutionize the treatment of NAFLD, Gall Stone disease, obesity and type 2 diabetes mellitus.

HDL particles participate in reverse cholesterol transport, the mechanism by which cholesterol from extrahepatic tissues returns to the liver for excretion as biliary cholesterol[44]. Low HDL-cholesterol is associated with metabolic syndrome and NAFLD[28]. ABCA1 is a lipid binding protein which increases reverse cholesterol transport to pre-beta HDL. Patients with low HDL-cholesterol and abnormal cellular lipid efflux due to ABCA1 gene defects (Tangier disease) also have elevated plasma triglycerides[44,54] and fatty liver.

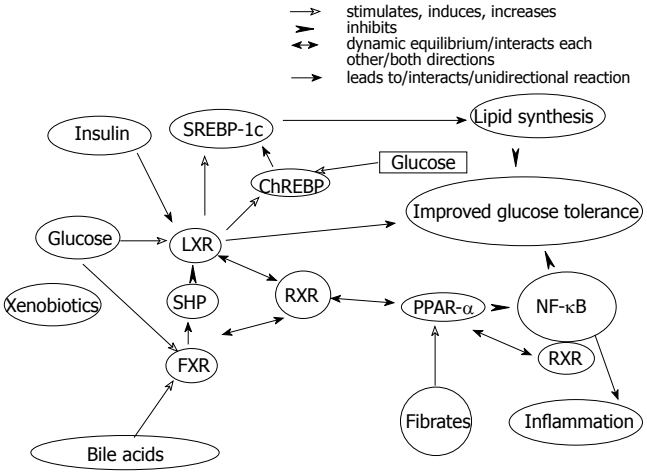


Figure 5 The complex interactions between various nuclear receptors involved in lipid-carbohydrate-bile acid homeostasis determines fat accumulation in liver. SREBP-Sterol regulatory element-binding proteins LXR-liver xenobiotic receptor, FXR-farnesoid xenobiotic receptor, SHP-short heterodimer partner, RXR-retinoid xenobiotic receptor, PPAR-peroxisome proliferator-activated receptor, ChREBP-carbohydrate responsive element binding protein. NF-κB-nuclear factor κB.

The increased synthesis

The synthesis of lipid and lipoproteins is important to metabolic disorders. In NAFLD nearly a quarter of the accumulated fat comes from *de novo* lipid (DNL) synthesis[17] and hepatic lipid synthesis is markedly increased in NAFLD[55]. Acetyl coenzyme A (acetyl CoA) is a crucial metabolic intermediate in carbohydrate and protein catabolism towards lipid synthesis. Acetyl CoA carboxylase (ACC) and Fatty Acid synthetase are two major enzymes that drive DNL in the liver[3]. Inhibitors of ACC decreases lipid accumulation by hepatocytes and might prove useful in the development of novel therapeutic agents to combat fatty liver[56].

Synthetic pathways for triacylglycerol (TAG), cholesterol and its esters, and phospholipids are separate, but transcriptionally co-regulated[57]. In insulin sensitive tissues and particularly in the liver, the transcription factor SREBP-1c transduces the insulin signaling regulating lipid synthesis. Overexpression of nSREBP-1c in the liver of transgenic mice bypasses insulin requirement and activates the same genes stimulated by insulin and produces a triglyceride-enriched fatty liver with no increase in cholesterol. The mRNAs for fatty acid synthetic enzymes and rates of fatty acid synthesis are elevated fourfold in liver[57,58]. Alcohol induces fatty liver partly by impairing PPAR-α and PPAR-γ activity and activation of SREBP-1[59]. Overexpression of nSREBP-2 in liver increases the mRNAs encoding all cholesterol biosynthetic enzymes[57,60].

Under physiological conditions, SREBP-1c is transiently

induced in the liver by insulin through activation of IRS-2; this causes a switch from glycogen synthesis to lipid synthesis. To complete a feedback loop, SREBP-1c then suppresses IRS-2 transcription. Under certain pathogenic conditions, expression of SREBP-1c in the liver remains elevated, and this increases lipid synthesis with resultant accumulation of fat[61].

Hepatitis B virus (HBV) infection is associated with fatty liver in a significant proportion of patients. HBV encoded protein HBx causes lipid accumulation in hepatic cells which is mediated through SREBP1 and PPAR-γ[62].

Obesity and insulin resistance is a pro-inflammatory state characterized by increased levels of pro-inflammatory cytokines[27,63]. Cytokines like IL-6 and TNF-α further promotes insulin resistance by increasing hepatic suppressors of cytokine signaling (SOCS) expression[3,63,64]. Over expression of SOCS-1 and SOCS-3 in liver causes insulin resistance and an increase in the key regulator of fatty acid synthesis in liver, SREBP-1c. In obesity, increased SOCS proteins enhance SREBP-1c expression by antagonizing STAT3-mediated inhibition of SREBP-1c promoter activity[64]. Interestingly n-3 PUFAs downregulate SREBP 1-c, which increases transcription of genes responsible for fatty acid synthesis such as fatty acid synthase and stearoyl Co-A desaturase[65].

The liver has a central role in glucose homeostasis. On feeding, glucose influx triggers gene expression changes in hepatocytes to suppress endogenous glucose production and convert excess glucose into glycogen or fatty acids to be stored in adipose tissue. This process is controlled by insulin, although debate exists as to whether insulin acts directly or indirectly on the liver. Transcriptional activation of glycolytic and lipogenic genes requires the presence of both insulin and glucose, neither of which is active alone. Recently, carbohydrate responsive element binding protein (ChREBP) emerged as a pivotal transcription factor implicated in the regulation of lipogenic genes by glucose[66].

Liver xenobiotic receptor (LXR) is another glucose sensor which is activated by glucose and switches on several genes involved in fatty acid synthesis[67]. An LXR-binding site in the *SREBP-1c* promoter activates SREBP-1c transcription in the presence of LXR agonists. When lipogenesis is increased by pharmacological activation of the liver X receptor, hepatic VLDL production is increased 2.5-fold, and the liver produces large TG-rich VLDL particles[68]. Interestingly, glucose induces expression of LXR target genes involved in cholesterol homeostasis like ABCA1 which is defective in Tangier disease[67].

A common feature of many metabolic pathways is their control by retinoid xenobiotic receptor (RXR) heterodimers. It is interesting to note that LXR heterodimerizes with RXR. Promiscuous RXR also heterodimerizes with PPAR members. PPAR-α plays a pivotal role in fatty acid catabolism in liver by upregulating the expression of numerous genes involved in mitochondrial fatty acid oxidation. Thus RXR is a common partner of two nuclear receptors acting in opposite directions with regard to fatty acid metabolism. So both LXR and PPAR-α compete for the limited pool of RXR and this dynamic equilibrium determines the direction of lipid metabolism[69].

FXR also plays a key regulatory role in glucose homeostasis. FXR-null mice developed severe fatty liver and elevated circulating FFAs, which was associated with elevated serum glucose and impaired glucose and insulin tolerance[70]. Activation of FXR lowers plasma glucose levels in fasted, fed, diabetic mice. Bile acids, by activating FXR, induce the expression of short heterodimer partner (SHP)[71]. SHP then interferes with SREBP-1c expression by inhibiting the activity of LXR and eventually other transcription factors that stimulate SREBP-1c expression[47].

An increase in serum triglyceride concentrations and fatty liver have been observed in patients with malabsorption of bile acid as found in chronic inflammatory bowel disease[72], ileal resection and cholestyramine treatment. This could be due to loss of bile salt mediated inhibitory effect on fatty acid synthesis mediated through FXR and further research is required in this area.

It may be noted that NAFLD is classically associated with gall stone disease and hypertriglyceridemia[30,73]. The increased incidence of gall stones at least partly is due to decreased secretion of bile salts, which are potent emulsifiers, and the consequent instability of bile pigments resulting in precipitation and stone formation. It would be logical to hypothesize that defective FXR expression or signaling and consequent deficiency in bile inhibition of fatty acid synthesis might play a role in certain cases of hepatic steatosis associated with biliary stones.

Hepatocyte nuclear factor-4α (HNF-4α) is a transcription factor which is mutated in monogenic autosomal dominant non-insulin-dependent diabetes mellitus type 1 (MODY-1), controls the expression of several genes, including hepatocyte nuclear factor 1α (HNF-1α), a transcription factor which regulates the expression of several hepatic genes and the human CYP7A1 gene in bile acid synthesis and phosphoenolpyruvate carboxykinase (PEPCK) gene in gluconeogenesis[74]. Long-chain fatty acids, including palmitic acid, have been identified as endogenous HNF-4α ligands and this allows the transcriptional control of gluconeogenesis during active lipid synthesis. It is interesting to note that glucose and fructose induces lipogenesis reduces hepatic HNF-4α levels, which in turn attenuates the expression of sex hormone binding globulin (*SHBG)*, a biomarker of metabolic syndrome[75]. Thus HNF-4α is another major protein at the cross roads of sex hormones, diabetes, fatty liver, dyslipidemia and gall stone disease.

Nuclear receptors are notoriously promiscuous. They are known to be activated, inhibited or otherwise modulated by numerous xenobiotic compounds which include alcohol, drugs, insecticides, dietary contaminants and numerous other chemicals acquired from environment[76]. This could explain the association of certain drugs as well as environmental toxins with fatty liver[77,78]. It may be noted that many of the *de novo* lipid synthesis pathways described above are shared by both alcoholic and nonalcoholic fatty liver disease.

The decreased oxidation

Fatty acid oxidation is compartmentalized in eukaryotic organisms into three subcellular organelles, with beta-oxidation confined to mitochondria and peroxisomes

and CYP4A-catalyzed omega oxidation occurring in the endoplasmic reticulum. Beta oxidation is primarily involved in the oxidation of fatty acids with carbon chains not longer than twenty while peroxisomal oxidization predominantly deals relatively complex and toxic fatty acids containing twenty or more carbon atoms and C27-bile acid intermediates[79]. Microsomal omega oxidation system hydroxylates saturated and unsaturated fatty acids which will eventually be oxidized by the other two oxidation systems[79]. Some of the key enzymes of these three fatty acid oxidation systems in liver are regulated by PPAR-α. When mice fed on a methionine-choline deficient (MCD) diet-a dietary model of fibrosing steatohepatitis-were treated with the PPAR-α agonist, Wy-14643, developed significantly less steatohepatitis, markedly lower ALT levels and less lipid peroxidation compared to controls. This occurred despite a marked increase of the liver P-450 enzymes which are oxidant stress inducers[80]. Mice lacking PPAR-α (PPARα-/-) fail to respond to the inductive effects of peroxisome proliferators, whereas those lacking fatty acyl-CoA oxidase (AOX-/-), the first enzyme of the peroxisomal beta-oxidation system, exhibit extensive microvesicular steatohepatitis, leading to hepatocellular regeneration and massive peroxisome proliferation, implying sustained activation of PPAR-α by natural ligands[79]. Ethanol a common cause of fatty liver interferes with DNA binding and transcription-activating properties of PPAR-α, as demonstrated with cultured cells and in ethanol-fed mice. Treatment of ethanol-fed mice with a PPAR-α agonist can reverse fatty liver even in the face of continued ethanol consumption[81].

Mitochondrial beta oxidation results in shortening of fatty acids and FA are oxidized carbon by carbon into acetyl-CoA subunits, which either condenses into ketone bodies that serve as oxidizable energy substrates for extrahepatic tissues, especially during starvation, or enter into the tricarboxylic acid cycle. Mitochondrial beta oxidation is regulated by carnitine palmitoyl transferase-1 (CPT-1), the carnitine concentration and malonyl-CoA, which inhibits CPT1. Fatty acids, fatty acyl-CoAs and several structurally different chemicals known as peroxisome proliferators, which activate PPAR-α, regulate CPT1 levels in the liver[82]. Inhibition of fatty acid oxidation in the liver is a intrahepatic cause of the development of liver steatosis. For instance, etomoxir, a CPT-1inhibitor, inhibits fatty acid oxidation and induces steatosis[83].

Thioredoxin (TRX) is an important redox regulator protein that has a redox-active dithiol/disulfide in its active site[84]. TRX binding protein-2 (TBP-2) was identified as a TRX binding protein from a yeast two-hybrid system study, and as a negative regulator of TRX through direct interaction. TBP-2 null mice are viable and fertile, but under fasting conditions, their survival rate was sharply reduced, concomitant with fatty liver, hypoglycemia, dyslipidemia, severe bleeding and hepatic and renal dysfunction. This mouse has been proposed as an animal model for Reye's syndrome an acute illness which is encountered exclusively in children below 15 years of age characterized clinically by vomiting and signs of progressive central nervous system damage, hypoglycemia and extensive fatty liver and liver damage[84]. TBP-2 is also reported to be a causative gene for familial combined hyperlipidemia (FCHL) in mice. Individuals with genetic deficiencies in fatty acid transport and mitochondrial oxidation show a similar pathology to Reye syndrome, which is defined as a Reye-like syndrome, such as CPT-I deficiency[85]. Defects in different enzymes involved in fatty acid utilization give rise to different disorders. A defect in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) is associated with acute fatty liver of pregnancy (AFLP) while medium-chain acyl-CoA dehydrogenase (MCAD) defect is involved in sudden infant death syndrome (SIDS)[86].

Less is understood about the role of mitochondrial beta oxidation in development of NAFLD. Mitochondrial trifunctional protein (MTP) catalyzes long-chain fatty acid oxidation. Chronic alcoholism is known to cause mitochondrial damage[87]. Megamitochondrion with crystalline inclusions is a feature of NAFLD underscoring its importance in pathogenesis[88]. A mouse model for MTP deficiency reported that homozygous (*MTPa*-/-) mice suffer neonatal death. Heterozygosity for fatty acid oxidation defects predisposes to NAFLD and insulin resistance in aging mice. Thus impaired mitochondrial oxidation may play an important role in pathogenesis of NAFLD[89]. Primary or age-related defects in mitochondrial beta oxidation could play a role in development of NAFLD[90]. MTP defects in man are recessively inherited and children with defects of any of the three enzymatic functions exhibit mostly microvesicular hepatic steatosis[91].

A diet that provides 2%-5% of energy from (n-3) and (n-6) PUFA leads to a coordinate suppression of glycolytic and lipogenic genes and to an induction of genes involved in fatty acid oxidation. This metabolic balance in liver leads to a 'partitioning' of fatty acids away from triglyceride synthesis toward fatty acid oxidation[92]. Long-chain n-3 PUFAs upregulate PPAR-α, increases transcription of genes responsible for fatty acid oxidation, such as mitochondrial CPT 1 and peroxisomal acyl-CoA oxidase[65]. Thus PUFA intake could be beneficial in treatment of NAFLD.

Epigenetic factors

The thrifty phenotype hypothesis (Barker's hypothesis) postulates epigenetic memory of the fetal/neonatal environment[93]. Feeding rats a high-carbohydrate diet during suckling rapidly leads to hyperinsulinism. Moreover, when these female rats become pregnant, hyperinsulinism also rapidly appears in their offspring even though they are fed a normal diet, suckling milk from their mothers[94]. Epigenetic regulation of gene expression involves chemical modification of chromatin by enzymes such as sirtuins (SIR2-related proteins), whose activities are linked to cellular energy stores and in lower organisms, sirtuin signaling interface with insulin signaling pathways[95,96] and SIR2 orthologues could possibly be involved in the pathogenesis of fatty liver disease.

Timers of fatty liver disease

Many biological processes are timed and there is a biological clock which keeps the time at the molecular level. Recent

studies have revealed that endogenous rhythms are generated at the cellular level by circadian core oscillators, which are composed of transcriptional/translational feedback loops involving a set of *CLOCK* genes. Obese diabetes attenuated this rhythmic expression in most of the clock and adipocytokine genes in adipose tissue. There was impairment in the rhythmic expression of the *CLOCK* genes in the liver as well. Interestingly, pioglitazone treatment improved the attenuated rhythmicity in the liver but not the adipose tissue[97]. A recent human study suggests a potential role of the *CLOCK* gene variants and their related haplotypes increased susceptibility to NAFLD and disease progression[98].

FATTY LIVER: THE MANY FACES IN CLINICS

The classic 'metabolic syndrome associated NAFLD'

The 'classical NAFLD' which is the 'fatty liver of affluence' is the commonest form and is associated with metabolic syndrome and can be viewed as a special class of adipose tissue malfunction and disordered lipid homeostasis. MS can be consider MS as inappropriate expression of 'thrifty genes' resulting in obesity, abnormal fat deposition, dyslipidemia, diabetes, pro-inflammatory (and pro-neoplastic?) states[99]. NAFLD, which is characterized by abnormal fat deposition in liver and chronic pro-inflammatory state, has been considered as a liver manifestation of MS[100]. This is substantiated by the epidemiological data where 50%-80% of the NAFLD has associated MS depending on the area studied and methods adopted. The association of NAFLD with MS and IR has been extensively reviewed by Marchesini *et al* and Cortez-Pinto *et al*[4,100].

Metabolic Syndrome is a tool for identifying individuals who are at the cardiovascular risk rather than a single disease. NAFLD is gaining importance in this context. Recent studies have reported the association of NAFLD with multiple classical and non-classical risk factors for cardiovascular disease (CVD). Moreover, there is a strong association between the severity of liver histopathology in NAFLD patients and greater carotid artery intima-media thickness and plaque, and lower endothelial flow-mediated vasodilation (as markers of subclinical atherosclerosis) independent of obesity and other MS components[28-30,101,102].

Identification of MS is very useful not only as a tool to identify people with cardiovascular and diabetic risk but also NAFLD but much confusion exists in the criteria of MS[103]. Criteria for MS should be convenient but flexible to the regional and ethnic differences is important in establishing its association with NAFLD, a disease which progress asymptomatically to cirrhosis[104]. There exists significant genetic environmental and life style diversity in population across different continents and cultures. For example Asian Indians are prone to central obesity and for same degree of weight gain are more insulin resistant[105-107].

In case of other well known syndromes, a single or a few well defined genetic defects results in the clustering of a host of strongly associated phenotypic features. But in MS, a host of poorly defined genetic defects results in loosely associated clustering of phenotypic features. The primary underlying causes of the metabolic syndrome are thought to be insulin resistance. Central obesity almost certainly is a major cause of insulin resistance[103]. There is considerable doubt whether all patients with the metabolic syndrome are indeed insulin resistant[103]. Many nondiabetic adults with a wide range of age and body mass are hyperinsulinemic and insulin resistant (about 50%), approximate 25% are insulin resistant but without hyperinsulinemia and the same proportion are hyperinsulinemic but without insulin resistance[103,107]. Thus the lack of consensus regarding MS criteria, insulin resistance cut off value together with the complex pathogenesis of hepatic steatosis makes it difficult to evaluate their association.

The lean nonalcoholic fatty liver disease

It became clear that Non-alcoholic steato hepatitis (NASH) has an equal sex distribution and that many, perhaps even the majority of patients according to some reports are neither obese nor diabetic[108].

We already described the importance of adipose tissue in fatty acid buffering. Several congenital forms of lipodystrophy are associated with fatty liver. Berardinelli-Seip syndrome caused by mutation of *BSCL2* gene is characterized by a near-total lack of body fat from birth. Interestingly *BSCL2* gene is highly expressed in the brain but only modestly in adipocytes, suggesting a role for the central nervous system in the pathogenesis[109]. MRI studies also reveal a near-complete absence of metabolically active adipose tissue from most subcutaneous areas, intraabdominal and intrathoracic regions and bone marrow. Fatty liver has been noted during infancy and can lead to cirrhosis and its complications. They show extreme insulin resistance[110].

Dunnigan variety of familial partial lipodystrophy and Mandibuloacral dysplasia (type-A lipodystrophy) are associated with laminin gene mutation, causes fatty liver and IR. PPAR-γ mutations result in a dominant form of lipodystrophy and fatty liver. Similarly in acquired lipodystrophies like Lawrence syndrome; hepatomegaly due to fat infiltration is a consistent finding[110].

In patients who undergo liposuction or other forms of obesity reduction surgery, fatty liver is a common finding. Highly active antiretroviral therapy (HAART) therapy for HIV, a combination that includes HIV-1 protease inhibitors, is associated with the development of lipodystrophy in the majority of patients after 18 mo to 2 years of treatment. It is characterized by fatty liver following marked reduction in subcutaneous fat from the face, trunk and limbs, resulting in an appearance of "increased muscularity". These patients are prone to develop insulin resistance, hypertriglyceridemia and fatty liver[111]. In animal models of lipodystrophy IR and fatty liver are corrected by infusion of adipokines[112]. This underscores the role of adipokines in modulating the response of adipose tissue and as messengers to other organs in response to changing energy environment and adaptive mechanisms like IR. Removal of adipose tissue causes IR and fatty liver in hamsters[113]. It is well known that patients who undergo liposuction as a treatment for

obesity do not show metabolic benefits[21]. It won't be surprising if liposuction worsens insulin resistance and fatty liver as this procedure removes active adipocytes from subcutaneous spaces. Thus, any defect that prevents adipose tissue from acting as an 'energy rich substrate sink' would result in fatty liver.

The fatty liver associated with infectious and immunological disorders

Fatty liver is associated with many chronic inflammatory conditions. Hepatitis C especially type 3 genotype is associated with fatty liver in nearly 50% of the patients. In patients with chronic liver disease due to hepatitis C virus, the major adipokine adiponectin was positively correlated with hepatic inflammation and adiponectin receptors were differentially regulated in the setting of hepatic insulin resistance[114]. It has been shown, that HCV internalization is facilitated *via* LDL (low density lipoprotein) receptors and the virus enters into the cell via endocytosis. More recently, a broadly expressed lipoprotein binding receptor, the human scavenger receptor class B type 1 was shown to serve as a receptor for HCV[115]. According to Younossi the HCV genotype 3 core protein inhibits very low-density lipoprotein (VLDL) secretion, leading to hepatic steatosis. Several studies demonstrate that steatosis disappears after patients with hepatitis C genotype 3 achieve a sustained response. The resolution of steatosis after treatment strongly supports the association between hepatic steatosis and HCV genotype 3[116].

Though less frequently, Hepatitis B is also associated with fatty liver. The hepatitis B virus encoded protein, HBx causes lipid accumulation in hepatic cells which mediated is by SREBP1 and PPARγ[117].

Fatty liver has been reported with certain bacterial infections like Q fever[118].

Autoimmune diseases like systemic lupus erythematosis (SLE) and rheumatoid arthritis *etc* are also associated with fatty liver[119].

There are reports that NAFLD is characterized by a low-grade systemic inflammation[120]. This could be partly because of the association of NAFLD with obesity which is a pro-inflammatory state. Conditions that induce inflammatory state in liver might in long term stimulate adipocytes and macrophages because they have a common ancestry and hence they share many cytokines, growth factors and signaling pathways[8-11,13]. There are many ways by which fat is deposited in liver but it is the body's response to fat and its derivatives which determines the progress of the disease from simple fatty liver to steatohepatitis and cirrhosis.

The xenobiotic fatty liver

NASH features are also encountered as an adverse reaction to a few drugs-for example, amiodarone and perhexiline. Drug like methotrexate, aspirin, vitamin A, glucocorticoids, amiodarone and synthetic estrogen causes macrovescicular steatosis while microvesicular is caused by valproic acid, tetracycline, nucleoside analogues etc. Chronic alcoholism causes predominantly macrovescicular steatosis[121,122]. However some hepatocytes in these livers with microvesicular steatosis may also reveal a macrovesicular fatty change, implying that with the progression of disease some of these small lipid vacuoles may fuse to become a large droplet.

The metabolism of ethanol enhances the level of NADH in the liver which, in turn, stimulates the synthesis of fatty acids and their incorporation into triglycerides. Ethanol mediated impairment or inhibition of PPAR-α and PPAR-γ and stimulation of SREBP, the receptor molecules that control the enzymes responsible for the oxidation and synthesis of fatty acids, respectively, appear to contribute to the overall lipid load in the alcoholic liver[59].

These xenobiotics cause steatosis by a host of mechanisms. Drugs like tetracycline, aspirin etc do so predominantly by inhibiting hepatic fatty acid oxidation[49]. Jamaican vomiting syndrome which is caused by hypoglycin-A present in unripened ackee fruit[121,123] is an example for natural toxins inducing fatty liver. Estrogen, various pesticides etc probably induces various nuclear receptors like FXR, PXR, CXR, LXR, PPARs *etc.* involved in lipid homeostasis as these nuclear receptors are promiscuous[76,77]. There is a paucity of literature and much research has to be done in this field.

Reye's syndrome and Reye like syndrome associated fatty liver

This acute illness is encountered exclusively in children below 15 years of age classically following a viral infection and the use of aspirin. It is characterized clinically by vomiting and signs of progressive central nervous system damage, signs of hepatic injury and hypoglycemia. Morphologically, there is extensive fatty vacuolization of the liver and renal tubules. There is mitochondrial dysfunction with decreased activity of hepatic mitochondrial enzymes. There are reports linking Thioredoxin Binding Protein-2, a protein that interacts with thiredoxin which is an important redox regulator protein and Rye's syndrome. Individuals with genetic deficiencies in fatty acid transport and mitochondrial oxidation show a similar pathology to Reye syndrome, which is defined as a Reye-like syndrome, such as CPT deficiency[84,85].

The fatty liver of pregnancy

Acute fatty liver of pregnancy (AFLP) is a syndrome that occurs late in pregnancy and is often associated with jaundice and hepatic failure. AFLP has an incidence of 1 per 13000 deliveries. It affects women of all ages and races. The liver is typically small. AFLP is more common when the mother is carrying a male fetus and associated with a deficiency of long-chain-3-hydroxy acyl COA dehydrogenase (LCHAD). Preeclampsia or the HELLP syndrome, which may complicate eclampsia, presents in a similar fashion and progresses to severe liver dysfunction, though typically with a normal size liver. Aminotransferase elevations are typically modest in all of these conditions[124].

An association between recurrent maternal acute fatty liver of pregnancy with a fetal fatty acid oxidation disorder was reported first in two siblings who both died at 6 mo of age[125]. In another study involving 11 pregnancies in 5 mothers where 6 babies had confirmed deficiency of LCHAD, by enzymatic analysis of cultured skin fibroblasts. The mothers had either AFLP or HELLP

Table 1 Fatty liver: the many faces in clinics

Classification of fatty liver	
Classic metabolic syndrome associated fatty liver	'Fatty liver of the affluent': commonest form of fatty liver; associated with metabolic syndrome
The lean nonalcoholic fatty liver	Includes lipodystrophies, known and unknown adipose tissue defects of various origin resulting in lean phenotypes, liposuction (?)
Fatty liver of infection/ chronic inflammatory diseases	HCV, HBV, Q fever (?) autoimmune diseases
Xenobiotic fatty liver	Drugs like tetracycline, amiodarone, alcohol, environmental industrial toxins
Fatty liver of defective fatty acid catabolism	Reyes syndrome, reye like syndrome, sudden infant death syndrome, fatty liver of pregnancy
Malnourishment associated fatty liver	'Fatty liver of the deprived' : kwashiorkor
Transient fatty liver	Fatty liver which develops transiently following short excess calorie intake

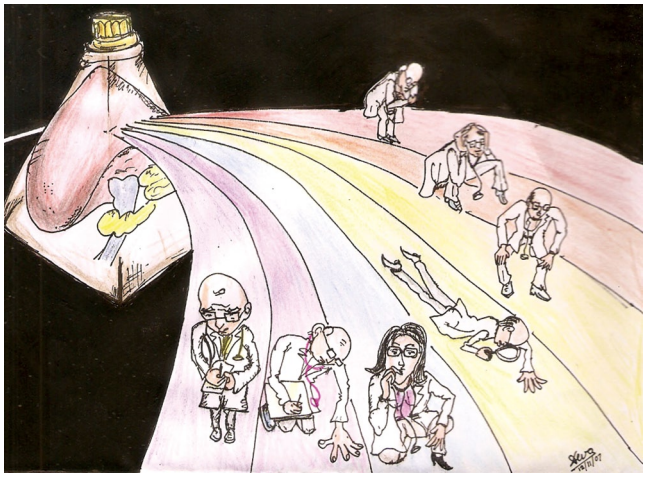


Figure 6 With the progress of medicine at molecular level diseases like type 2 diabetes and 'NAFLD' which results from a spectrum of genetic defects would split into hundreds if not thousands of diseases with 'individual' identity providing patients' accurate diagnosis of the disease and accurate treatment with minimum 'side effects' which is unavoidable in today's 'blind clinical practice'.

syndrome in all six pregnancies with the LCHAD deficient fetuses[126]. However how fetal defects induce acute fatty liver in mothers is still not clear.

Fatty liver in malnutrition

Protein malnutrition, especially in infancy and early childhood, accounts for most cases of severe fatty liver in the tropical zones of Africa, South America, and Asia. The hepatic changes may be associated with other clinical and pathologic features of Kwashiorkor. High plasma levels of free fatty acids are generally regarded as a key biochemical feature of the condition. Glucose intolerance is another characteristic feature. Interestingly in marasmus, a form of protein-calorie malnutrition, fatty liver is not part of the clinical picture[127].

The benign fatty liver-the non-progressive fatty liver

Kagansky *et al* evaluated 91 octogenarians (mean age, 85.56 ± 3.76 years) to determine the prevalence and the clinical presentation of NAFLD in the elderly. Among these patients, who had been admitted to a geriatric hospital, about 50% were diagnosed with NAFLD. An association of NAFLD with the metabolic syndrome or advanced liver disease was not observed in this older patient population. These data indicate that NAFLD is a common and relatively benign finding in the elderly[128]. Another study, examined sequential liver biopsies obtained from 103 NAFLD patients. The mean interval between biopsies was about 3 years and found that the stage of fibrosis slowly progressed in 37% of patients but remained stable in 34%[129]. The observation that NAFLD patients without NASH have a benign prognosis confirmed in several other studies as well[130].

Transient fatty liver

It is possible that fatty liver is physiological than pathological under certain circumstances for example after a fat carbohydrate rich diet for a short period. Increase in hepatic TG content can occur within 10 d after starting the high-fat diet in mice[131]. Overnight fasting increases plasma FFA to such an extent that liver TG content increases in dogs[132]. It is logical to assume that as in laboratory animals transient fatty liver follows a short term calorie rich diet in man as well. Unfortunately there is no diet related guide line for ultrasonological determination for fatty liver in the diagnosis of NAFLD. Ideally the subject should be on 'normal' and balanced diet at least for a week prior to ultrasonological evaluation for NAFLD. In a large population based study involving 4401 subjects it was observed that fatty liver regressed in a significant number of participants; fatty liver regressed in 14% of men and 25% of women[133]. In another follow up study involving sequential liver biopsies from 103 NAFLD patients it was observed that disease regressed in 29% of the cases[129]. In another recent study fatty liver regressed in nearly 1 of every 2 cases and had a substantially benign course[134]. A carbohydrate rich but fat deficient diet may predispose to fatty liver as surplus carbohydrate induce adipogenic genes[135]. This could well explain the fatty liver, which follows total parenteral nutrition[136].

The classification of fatty liver is summarized in Table 1.

NEED FOR PERSONALIZED MANAGEMENT OF NAFLD: NO TWO PEOPLE ARE THE SAME!

The advances in science would soon empower us to understand subtle differences between two individual patients at genotypic and phenotypic levels. It seems likely that a major focus of medicine over the next two decades will be within the arenas of 'pathogenomics', molecular diagnostics and 'pharmacogenomics' as a prelude to personalized molecular medicine. All polygenic

diseases like type 2 diabetes and 'NAFLD' would split into hundreds if not thousands of diseases with 'individual' identity providing patient' accurate diagnosis and accurate and effective treatment with minimum 'side effects' which is unavoidable in today's 'blind clinical practice' (Figure 6).

The difference in genotype and environment could explain the differences in the results observed even between drug well controlled drug trials and why certain drug therapies are effective only in certain individuals.

Above all, molecular level diagnosis and personalized treatment is more important because of difference in life style and environmental factors which are detrimental in the disease progress and treatment options. As mentioned before interactions between the genotype, phenotype and environment determines the disease process.

CONCLUSION

Currently the term NAFLD is loose to incorporate a host of diseases presenting with fatty liver in the absence alcohol abuse. 'Insulin resistance associated fatty liver' is the commonest type which could result from a variety of genetic, epigenetic defects and environmental factors. Clinically fatty liver is an important sign in predicting the risk of diabetes, cardiovascular accidents, stroke, cirrhosis and cancer. Individual variability in evaluating alcoholism and histopathological specimens, the variety of genetic defects presenting as fatty liver, together with the absence of reliable disease markers makes the accurate diagnosis of NAFLD difficult. Fat accumulation results from imbalance between feeding and physical activity, inflow and out flow, synthesis and oxidation. At molecular level defects in nuclear receptor signaling involving lipid sensing, synthesis and oxidation, defects in the lipid influx-efflux channels, proteins involved in insulin signaling, fatty acid catabolism, molecular defects in adipose tissue development, maturation and function and neural signaling can cause steatosis.

Bundling of several genetic defects under same diagnosis can result in inefficient patient management. With the advances in molecular medicine and diagnostics, diseases like NAFLD and Diabetes Mellitus Type-2 which are currently considered and managed as single entities would split into several distinct entities which might differ in management. Thus exploration of the molecular and genetic basis of NAFLD will allow us to segregate and manage them appropriately.

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REVIEW

Evidence for the involvement of infectious agents in the pathogenesis of Crohn's disease

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Abstract

Many advances have been made in the understanding of Crohn's disease (CD) pathogenesis during the last decade. CD is currently seen as a predominantly T-lymphocyte-driven disease characterized by the presence of a complex cocktail of interacting cytokines, chemokines and other mediators produced by a variety of cell types. Prevailing theories of CD pathogenesis suggest that patients' T-lymphocytes are inappropriately activated in the setting of an immune imbalance, which is itself caused by an unfortunate confluence of genetic and environmental factors. The T-cell response then leads to the chronic inflammation characteristic for the disease. Various environmental factors may play a role in the development of CD, but microbes are most consistently implied. This theory is based on epidemiological, clinicopathological, genetic and experimental evidence. Despite the abundance of arguments for the implication of bacteria in the etiopathogenesis of CD, the precise role of bacteria in this disease still remains elusive. Three not necessarily mutually exclusive theories have been proposed: (1) an unidentified persistent pathogen; (2) an abnormally permeable mucosal barrier leading to excessive bacterial translocation; and (3) a breakdown in the balance between putative "protective" versus "harmful" intestinal bacteria ("dysbiosis"). At present, one cannot exclude with certainty any of these three proposed hypotheses; they may all apply to CD to a certain extent.

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Key words: Crohn's disease; Etiology; Microbiology

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INTRODUCTION

Many advances have been made in the understanding of Crohn's disease (CD) pathogenesis during the last decade. CD is currently seen as a predominantly T-lymphocyte-driven disease characterized by the presence of a complex cocktail of interacting cytokines, chemokines and other mediators produced by a variety of cell types. Prevailing theories of CD pathogenesis suggest that patients' T-lymphocytes are inappropriately activated in the setting of an immune imbalance, which is itself caused by an unfortunate confluence of genetic and environmental factors. The T-cell response then leads to the chronic inflammation characteristic for the disease.

MICROBES, AN ENVIRONMENTAL FACTOR IMPLIED IN CD PATHOGENESIS

Various environmental factors such as smoking, appendectomy and the composition of the diet may play a role in the development of CD[1-6]. Microbes are however the most consistently implied factor in CD pathogenesis. This theory is based on epidemiological, clinicopathological, genetic and experimental evidence.

Epidemiological evidence

Evolutions in the epidemiology of CD during the second half of the 20th century may be explained in part by changes in the exposure of the human intestine to microbes over the same period. Some investigators propose that increased hygiene and the concomitant delayed exposure to enteric pathogens have contributed to the surging incidence of CD[7]. Not only the timing but also the type of microbes to which the gut is exposed may be important[8]. Changing dietary practices such as decreased consumption of oats, rye and bran, increased consumption of uncooked pork, unpasteurized milk and cheeses, and drinking well water instead of tap water may all favour a shift from a "protective" to a "harmful" gastrointestinal (GI) flora[9]. The "cold chain hypothesis" postulates that

CD results from an increased and chronic exposure to microbes which are able to survive at low temperatures (so-called psychrotropic bacteria, such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium botulinum* and *Bacillus cereus*). The chronic ingestion of these bacteria, new in the 20th century, would result from the increasingly common use of refrigeration in food preparation and storage, with the domestic refrigerator as the final step in the cold chain[10]. Another possible cause of sustained alterations in the gut microbiota is the increased use of antibiotics in human and veterinary medicine. Insufficient doses of antibiotics can induce a capacity for toxin production in bacteria or can make them invasive. It has been documented that prior antibiotic therapy can promote the outbreak of CD, and antibiotic use 2-5 years pre-diagnosis has an odds ratio of 1.32 in CD incident cases after adjusting for age, sex, smoking and the use of other drugs[11,12].

Clinicopathological evidence

The clinicopathological features of incident and recurrent CD, including its extra-intestinal manifestations, can be mimicked to a certain degree by specific enteric infectious diseases. Most notable in this regard are *Campylobacter*, *Salmonella*, *Shigella* and *Yersinia* infections. These can all present with an endoscopic picture of focal/segmental enterocolitis. *Yersinia* species (spp) may persist for a long time in the bowel wall and sustain chronic inflammation with its attendant consequences for tissue architecture. Differential diagnosis is by stool or blood culture or serology, as biopsies can show focal active colitis reminiscent of CD[13-22]. Other infectious microorganisms, which can cause diagnostic confusion, are *Escherichia coli (E. coli)* serotype O157:H7[23], *Chlamydia trachomatis*[24], *Mycobacterium tuberculosis*[25], *Actinomyces* spp[26], *Entamoeba histolytica*[27], *Giardia lamblia*[28], *Blastocystis hominis*, *Cryptococcus neoformans* and *Histoplasma capsulatum*.

Diarrhoea is a major symptom of CD, but also of infectious gastro-enteritis. This may occasionally cause differential-diagnostic problems. Infection with enteric viruses such as rotavirus, Norwalk-agent or adenovirus may provoke an exacerbation of pre-existing CD and thus enters in the differential diagnosis of CD relapse[29]. Acute bacterial enteritis can be confused with a first attack of CD, especially if stool cultures remain negative. Fulminant CD colitis with toxic dilatation, while being rare, is commonly treated surgically. These cases may be confused with infection by *Entamoeba histolytica*, *Salmonella* spp, *Campylobacter jejuni* and *Clostridium difficile*, which can sometimes be treated medically[30,31]. Chronic diarrhoea in immunocompetent patients is generally caused by non-infectious diseases like CD. The three most common infectious causes are *Giardia lamblia*, *Entamoeba histolytica* and *Clostridium difficile* (the latter can be provided with a niche by previous use of antibiotics). The clinicopathological manifestations in all three can be confused with CD. Clinical activity of CD may diminish with CD4 T-cell depletion in AIDS. On the other hand, very low CD4-counts may allow the development of AIDS-related GI infections[32]. Microscopic examination of a colonoscopic biopsy set may be helpful in the differential diagnosis between infectious gastro-enteritis and a first attack or relapse of CD. Infectious disease usually shows a picture of acute self-limited colitis, although this may evolve over time in focal active colitis. In such cases, differential diagnosis from CD becomes more difficult[33]. It should be noted that the typical histological features of chronic inflammatory bowel diseases (basal plasmocytosis, crypt distortion, villous colonic mucosa, crypt atrophy) increase in frequency with longer disease duration[34].

Infections may also arise as side-effects of CD therapy. Induction and maintenance of remission in active disease are usually obtained by suppressing the adaptive immune system. The attending risk is a re-activation of suppressed infectious agents or the creation of new opportunities for opportunistic pathogens. Examples of such infectious agents are *Clostridium difficile*, *Mycobacterium tuberculosis*, *Aspergillus spp*, cytomegalovirus, varicella-zoster virus and Epstein-Barr virus. The surgical treatment of CD can be complicated by abdominal wound sepsis (e.g. by *E. coli*, *Proteus spp* and *Staphylococcus aureus*), unless antibiotic prophylaxis was applied. The increased risk of surgical infections may be related to the high degree of bacterial translocation in CD[35].

It has been observed that CD recurrence in the neoterminal ileum after curative ileal resection depends on the presence of the faecal stream[36]. Histopathological and electron microscopic studies have shown that intestinal contents trigger postoperative recurrence proximal to the ileocolonic anastomosis already in the first days after surgery[37]. Metronidazole therapy for 3 mo decreases the severity of this early endoscopic recurrence and seems to delay symptomatic recurrence as well[38]. Likewise, ornidazole treatment for up to 1 year significantly reduces the endoscopic and clinical recurrence rate of CD after surgery[39]. The construction of a nipple valve may delay and perhaps prevent symptomatic recurrence[40]. A possible explanation is that the anastomotic configuration may influence colonization rates in the neoterminal ileum. When compared with controls, the neoterminal ileum in CD patients is more heavily colonized by *E. coli*, enterococci, bacteroidetes and fusobacteria[41].

Genetic evidence

In 2001, two independent groups identified the CARD15 gene on chromosome 16q as a susceptibility gene in CD. Three common single-nucleotide polymorphisms (SNPs) of this gene were found to be associated with development of CD: two missense mutations, Arg702Trp (SNP8) and Gly908Arg (SNP12), and a frameshift mutation, Leu1007fsinsC (SNP13)[42,43]. The CARD15 protein functions as an intracellular receptor for muramyl dipeptide (MDP), the minimal structural motif of peptidoglycan, a common component of gram-positive and gram-negative bacterial cell walls. MDP may enter the cytosol via the transporter protein hPepT1 and then binds to the C-terminal leucine-rich repeat (LRR) domain of the CARD15 protein[44]. Upon recognition of MDP, CARD15 activates the nuclear factor kappa B (NF-κB) pathway via a Rick/Rip2 interaction[45]. Because NF-κB is a key factor in the inflammatory response, CARD15 is considered as a pro-inflammatory molecule. The CD-associated mutations of the CARD15 gene affect the LRR domain of the protein and thus may impair its pro-inflammatory function. This

has potentially serious consequences given the normal expression of CARD15 in cells of the monocyte lineage and in Paneth cells, which are both involved in antibacterial defense[46-48]. The increased odds ratio for CD in double-dose CARD15 mutation carriers, the impaired defensin production by Paneth cells in ileal CD[49,50], data from *in vitro* models of transiently transfected cell lines[51-53] and a loss of response to MDP observed in *ex vivo* analyses of circulating monocytes from CD patients[54] all suggest a loss-of-function model for CARD15 in CD. It seems paradoxical that loss of function in a pro-inflammatory molecule such as CARD15 can cause an inflammatory condition such as CD. Most authors propose that defective recognition of bacterial components allows an increased bacterial proliferation in the gut, which in turn would be a powerful stimulus for the adaptive immune system. The end result might seem a T-lymphocyte-driven disease. Attractive as this theory is, it remains however at present wholly speculative. It should be noted that other explanations for the paradox have been offered as well[55-58].

Experimental evidence

There are currently more than 20 animal models of chronic inflammatory bowel disease. These models can be divided into 4 groups: (1) genetically induced as a result of deletion (e.g. $IL10^{-/-}$, T-cell receptor mutant mice) or overexpression of genes (e.g. IL7 transgenic mice, HLA-B27 transgenic rats); (2) adoptive transfer models (e.g. the CD45RB transfer model, the heat shock protein 60 CD8 T-cell transfer model); (3) inducible colitis models (e.g. TNBS- or DSS-induced colitis); and (4) spontaneous colitis models (e.g. C3H/HejBir mice, SAMP1/Yit mice)[59]. All the genetically engineered mouse models require commensal enteric bacteria for the development of intestinal inflammation. Various organisms including *Helicobacter* spp and *E. coli* have been implicated[60,61]. The role of the flora in the initiation of inflammation may be host-dependent. Development of inflammation in these animal models further requires a particular genetic background and the presence of T-helper cells[62].

THE PRECISE ROLE OF BACTERIA IN THE PATHOGENESIS OF CD

Despite the abundance of arguments for the implication of bacteria in the etiopathogenesis of CD, the precise role of bacteria in this disease still remains elusive. Three not necessarily mutually exclusive theories have been proposed: (1) an unidentified persistent pathogen; (2) an abnormally permeable mucosal barrier leading to excessive bacterial translocation; and (3) a breakdown in the balance between putative "protective" versus "harmful" intestinal bacteria ("dysbiosis").

The unidentified persistent pathogen theory

The similarities between CD and some forms of infectious enterocolitis are sufficiently evident that numerous specific microbial aetiologies for CD have been proposed over the years. One of the first descriptions of what later became known as "Crohn's disease"[63] was made by Dalziel in 1913[64]. He noted similarities between his cases of "chronic interstitial enteritis" in humans, and Johne's disease in cattle, which is caused by *Mycobacterium avium subsp paratuberculosis (MAP)*. He therefore suggested that *MAP* may have been the cause of the disease which he described. This hypothesis has since been tested extensively by numerous investigators, and evidence has accumulated both in favour and against the theory. *MAP* has been cultured from CD tissues[65] and peripheral blood of CD patients[66], although not uniformly[67]. The variability in the results may be partly due to the fastidious culture requirements of the cell wall-deficient forms ("L-forms") of *MAP*, which are reportedly present in CD. Detection rates for *MAP* DNA in CD tissues using a specific PCR range everywhere from 0% to 100%[68-70]. This large variation may be due to differences in DNA extraction techniques or to geographical variations in the prevalence and modes of transmission of *MAP*[71]. Investigation of the presence of an immune response to *MAP* antigens in CD patients has also yielded both positive and negative results[72,73]. Enthusiasm for *MAP* as the causative agent of CD has further diminished after the recent publication of the results of a large, prospective, placebo-controlled, double-blind, randomized trial of 2 years clarithromycin, rifabutin and clofazimine in active CD, with a further year of follow-up included in the study design[74]. Although after 16 wk treatment significantly more subjects were in remission in the antibiotic arm than in the placebo arm, this benefit could not be sustained later on. After two years, the relapse rate was similar in the antibiotic and the placebo group. The good result in the first 16 wk may have been due to concomitant administration of a tapering course of prednisolone, which can have non-specific bacterial effects. Numerous other specific bacteria have been proposed as candidate causative agents of CD, including *Pseudomonas maltophilia*[75], *Mycobacterium kansasii*[76], *Chlamydia trachomatis*[77], *Bacteroides fragilis*[78] and *Listeria monocytogenes*[79]. Like *MAP*, *P maltophilia* and *M kansasii* were first isolated from CD tissues as L-forms. This is by no means a rare phenomenon as a variety of other bacteria such as *Enterobacteriaceae*, *Staphylococcaceae* and *Streptococcaceae* have been cultured from tissues of IBD patients (but not from control subjects) as L-forms as well. Recovery rates of these L-forms are apparently not influenced by previous medical treatment. This suggests either that L-forms may be involved in disease causation, or that their presence in mucosal biopsies is a result of the disease process itself[80]. Adherent-invasive *E. coli* (AIEC) is a specific pathovar which has been isolated from the intestinal mucosa of CD patients. The reference strain LF82 is able to adhere to intestinal epithelial cells, to invade these cells via a mechanism involving actin polymerization and microtubules organization, and to survive and replicate within macrophages[81-83]. Recently, it was shown that pathogenic B2+D *E. coli* strains are significantly more abundant in IBD patients than in controls. The B2+D phylogenetic groups are associated with the presence of serine protease autotransporter proteins and adherence factors and may have a significant role in disease aetiology[84].

The excesssive bacterial translocation theory

Bacterial translocation can be defined as the passage of

viable endogenous bacteria from the GI tract to mesenteric lymph nodes and other internal organs. The proportion of surgically treated CD patients with bacterial translocation prior to antibiotics administration varies between 30% and 50%, which is much higher than in controls (5%-15%). This suggests that bacteria leak from the bowel lumen in a high proportion of CD patients. The translocating bacteria may be involved in the pathogenesis of fistulas and abscesses, and translocation may predispose patients undergoing laparotomy to the systemic inflammatory response syndrome. Bacterial strains involved in translocation include *E. coli*, *Enterococcus* spp, *Clostridium perfringens*, *Proteus* spp and *Bacteroides fragilis*. Distension of the intestine proximal to a stricture may predispose to bacterial translocation[85,86].

The dysbiosis theory

The human gut contains a large number of microorganisms, most of which belong to the domain Bacteria. The intestine of an adult human individual may contain more than 500 different bacterial species at any moment, with 30-40 species comprising up to 99% of the total population[87]. The concentration of bacteria increases along the small bowel from approximately 10^4 in the jejunum to 10^7 colony-forming units (CFUs) per g of luminal content at the ileal end, with predominance of gram-negative aerobes and some obligate anaerobes. The colon is heavily populated by strictly anaerobic bacteria at concentrations of approximately 10^{12} CFUs per *g* of luminal content[88]. The traditional method to investigate the composition of the gut flora is culture-based: bacteria are isolated on selective growth media and identified by phenotypic characteristics. However, over 50% of the bacterial cells observed by microscopic examination of a faecal specimen cannot be grown in culture. Molecular biologic techniques have recently been developed to characterize these bacteria. These new techniques are largely based on the sequence diversity of the 16S rRNA gene. This gene is essential for the survival of all bacteria, since it encodes a part of the ribosomal RNA. Its sequence contains well-preserved and more variable parts. The variability between the 16S rRNA gene sequences of two bacterial species can be used as a measure for their phylogenetic distance. Alternatively, the 16S rRNA gene sequence can be used to identify or characterize the bacteria present in a biological specimen, even if they are not cultivable. A large number of 16S rRNA gene sequences (> 79 000) are currently available in public databases[89].

Several studies using different methods have repeatedly shown that the faecal microflora differs between CD patients and healthy controls, although the reported changes are not always consistent[90]. Bowel contents in quiescent CD are characterized by high total viable counts, high biodiversity and temporal stability. There is an increased number of obligate anaerobic Gram-negative rods, e.g. *Bacteroides fragilis* and *B vulgatus*. Many unusual species are also present in low numbers. On the other hand, counts of butyrate-producing Firmicutes (especially those of the *Clostridium leptum* group) are typically diminished. This may lead to an increased frailty of the intestinal epithelium[91-97]. The observed changes are independent of disease location, the presence of anatomical abnormalities secondary to inflammation or scarring, treatment with sulphasalazine or corticosteroids and even surgery. It has therefore been suggested that the faecal flora abnormalities in CD may be due to constitutional factors[98-102]. CD relapse causes huge disturbances in faecal flora composition. With diarrhea, total viable counts diminish due to a decrease in the number of strictly anaerobic bacteria (e.g., bifidobacteria). There is also a shift towards facultative anaerobes (particularly *E. coli*)[103,104].

The alterations in the luminal or faecal flora in CD patients are however not necessarily relevant for what happens at the mucosal level. Most studies of the mucosal flora report an increased likelihood to obtain positive culture results and higher total viable counts in CD patients' biopsies than in controls, especially in the ileum. Mucosa-associated bacteria in CD typically form a thick, densely populated biofilm associated with the mucus which covers the intestinal epithelium. Bacterial concentrations are higher in normal-looking CD mucosa than in biopsies obtained from visibly inflamed areas[105-111]. Not only the abundance but also the composition of the mucosa-associated flora is abnormal in CD. In general, the prevalence of the phyla Proteobacteria and Bacteroidetes is increased at the expense of the phylum Firmicutes[112]. Facultatively anaerobic bacteria (enterobacteria and enterococci) are found more frequently in ileum biopsies from CD patients than in normal subjects[113]. Taken together, the literature data suggest that the mucosal flora in CD patients is abnormal even before the onset of inflammation[114].

CLINICAL IMPLICATIONS

Given the abundant evidence for a role of bacteria in the pathogenesis of Crohn's disease, it has been suggested that the intestinal bacterial flora can be manipulated for therapeutic purposes. Several investigators have reported on the use of antibiotics and probiotics in CD patients. From these data, it appears that treatments must be individualized, depending on disease location and activity[115].

Antibiotics

Antibiotic therapy in Crohn's disease may decrease the number of mucosa-associated bacteria, kill or suppress bacterial variants which induce early histological lesions, and reduce the rate of bacterial translocation. Broad-spectrum antibiotics are frequently and successfully used in the treatment of mild to moderate CD, although no large controlled trials have yet been performed[116].

Metronidazole has been most extensively investigated. This antibiotic is active against strictly anaerobic bacteria. It has been used in monotherapy, especially with colonic and ileocolonic CD, but not for ileitis[117-120]. It has however many side-effects such as nausea, anorexia, dysgeusia, dyspepsia and peripheral neuropathy, which may limit its use.

Other antibiotics have also been tested. Ciprofloxacin therapy is one of the potential treatments of active CD, and is also effective when used in combination with standard treatment in patients with resistant disease[121,122]. Combined ciprofloxacin plus metronidazole treatment has

been shown to be a potential alternative to steroid therapy in the acute phase of CD, although it is more effective in colonic than in isolated small bowel disease[123,124]. The combination of ciprofloxacin and metronidazole is also used in the treatment of perianal CD[125]. Other antibiotics which have been tested are rifaximin[126] and clarithromycin[127].

Probiotics

Probiotic therapy is one possible approach to the prevention of relapse in CD patients. Several microorganisms such as *E. coli* Nissle, *Lactobacillus GG* and *Saccharomyces boulardii* have been tested. At present however, there is no evidence that these probiotic preparations perform better than placebo or aminosalicylates in this setting. The use of probiotics as a maintenance therapy for medically or surgically induced remission in CD is therefore currently not recommended[128].

CONCLUSION

At present, one cannot exclude with certainty any of the three proposed hypotheses on the role of bacteria in CD etiopathogenesis. Uninflamed bowel mucosa in CD may be heavily colonized by facultative anaerobe and/or aerotolerant bacteria, possibly due to a genetically determined defect in the capacity of the mucosa to "hold back" such bacteria. The ecological conditions prevailing in this mucosa-associated biofilm may then enable some bacteria to acquire particular virulence traits by horizontal gene transfer. Eventually, pathovars may arise which have an increased capacity for adherence to and invasion of the mucosa. Aphtoid ulcerations may develop in this setting. Once the mucosal integrity is broken down, bacterial translocation through the bowel wall is greatly enhanced and may lead to the development of complications such as fistula and abscesses. The human body may then react with a basically appropriate but uncontrollable inflammatory response which leads to further tissue damage. While this disease causation theory stresses global disturbances in the composition of the bowel flora and increased bacterial translocation, it cannot be excluded that specific bacteria may be particularly damaging to people who are genetically susceptible to the development of CD. The adherent-invasive *E. coli* pathovar is perhaps the most likely candidate in this regard.

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REVIEW

Colonography by CT, MRI and PET/CT combined with conventional colonoscopy in colorectal cancer screening and staging

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Abstract

Colorectal cancer (CRC) remains a leading cancer killer worldwide. But the disease is both curable and preventable at an early stage. Regular CRC cancer screening has been shown to reduce the risk of dying from CRC. However, the importance of large-scale screening is only now starting to be appreciated. This article reviews a variety of imaging procedures available for detecting ulcerative colitis (UC) and Crohn's disease (CD), polyps and CRC in their early stage and also presents details on various screening options. Detecting, staging and re-staging of patients with CRC also require multimodality, multistep imaging approaches. Staging and re-staging with conventional colonoscopy (CC), computer tomography colonography (CTC), magnetic resonance colonography (MRC) and positron emission tomography/computer tomography colonography (PET/CTC) are of paramount importance in determining the most appropriate therapeutic method and in predicting the risk of tumor recurrence and overall prognosis. The advantages and limitations of these modalities are also discussed.

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Key words: Colon polyps; Colorectal cancer; Conventional Colonoscopy; Virtual colonoscopy; Computer tomography colonography; Magnetic resonance colonography; Positron emission tomography/computer tomography colonography

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INTRODUCTION

Over 55 000 Americans are expected to die of Colorectal cancer (CRC) in 2006[1]. It is estimated that there is over a 5% chance that an American will develop CRC in their lifetime and over a 2% chance that an American will die from CRC. In some Asian countries, the incidence of colorectal cancer rises rapidly[2]. Compared to Westerners, Chinese patients have a slightly lower prevalence of colon neoplasia, more distal distribution of neoplasia, and higher likelihood of concomitant proximal advanced neoplasia and distal neoplasia[3].

When used appropriately, screening for CRC can reduce disease-related morbidity and mortality[4]. Current methods include fecal occult blood testing (FOBT), flexible sigmoidoscopy, double contrast barium enema (DCBE), and conventional colonoscopy (CC); all are cost-effective techniques. Unfortunately, offering an array of options has not increased screening utilization, which continues to lag behind that of other common cancers[5]. Newer techniques, particularly virtual colonoscopy (VC), including CTC, MRC and PET/CTC may offer attractive alternatives for healthcare provider recommendation and patient use[6].

COLORECTAL CANCER RISK FACTORS

The exact causes of CRC are not known. However, studies show that the following risk factors increase a person's chances of developing CRC: over the age of 50, high fat and calories and low fiber diets, family history of colorectal cancer, previous history of CRC or adenomas, a cholecystectomy was performed ten years or more ago, severe ulcerative colitis (UC) and Crohn's disease (CD) for over ten years, *etc*[7-9].

There is substantial evidence that most CRC arise from preexisting adenomas. A large body of clinical evidence

Table 1 Characteristics of medical images in detecting CRC

Modalities	Characteristics
DCBE	Cost-effective, efficient, safe, could be performed and interpreted by GI radiographers to reduce waiting
Colonoscopy	Cost-effective, endoscopic resection of polyps, endomicroscopy opening the way of in vivo molecular imaging
CTC	Less uncomfortable, minimally invasive, with radiation exposure, 3D virtual dissection of colon,
MRC	Limited bowel preparation, unpleasant rectal tube, filling colon with saline and contrast, no radiation
PET/CTC	Accurate whole-body tumor staging, with integrated morphological and functional images, more irradiation

supports the belief that over 95% of CRC arise from benign adenomatous polyps that develop and grow very slowly over many years[10]. Consequently, polypectomy of colorectal adenomas was shown to reduce the incidence of CRC by nearly 80%. Progression of an adenoma into cancer can be predicted by its size, villous histology, degree of dysplasia, and inherited or environmental factors. Advanced adenomas are the primary target in colorectal screening. Advanced adenomas were generally large (> or = 10 mm in size); only a small percentage were medium sized (6-9 mm). There was a very low prevalence of high-grade dysplasia and invasive carcinoma in the medium-sized group of lesions[11]. So, there is a firm consensus that larger (> or = 10 mm) colonic polyps should be removed; however, the importance of removing smaller polyps (< 10 mm) is more controversial.

ULCERATIVE COLITIS OR CROHN'S DISEASE AND COLORECTAL CANCER

People with UC or CD have a high risk of developing CRC, even if the disease is in remission[12]. Regular screenings and early detection are critical. In people with UC or CD, CRC may be preceded by polyps or may arise from flat mucus membranes, thus requiring more intensive and more frequent colonoscopies[13]. CRC is more often multiple and uniformly distributed throughout the colon and often occurs in younger people with UC or CD[14]. CRC is one of the most serious potential results of IBD and explains up to one sixth of all deaths in UC patients and one twelfth of all deaths in CD[15]. Longer duration of the disease, extensive colitis, primary sclerosing cholangitis and family history of CRC are the main risk factors. Relative risk of CRC is 2.5 and small bowel cancer risk is 31.2 in CD[16]. A recent meta-analysis estimated that one in five patients with UC will develop CRC over 30 years, with the risk of CRC being greatest in patients with extensive UC of long duration. Patients with CD also have an increased risk of CRC[17]. Given this fact, it is necessary to early examine UC or CD by current and future approaches to CRC prevention in IBD patients[18].

COLORECTAL CANCER PROACTIVE SCREENING

CRC is generally more treatable when it is found early. CRC screening is used to detect cancer, precancerous polyps, or other abnormal conditions. If screening detects an abnormality, diagnosis and treatment can occur promptly. In addition, finding and treating polyps may be one of the most effective ways to prevent the development of cancer altogether. Because often there are no symptoms and symptoms do occur depend on the location of the cancer in the gastrointestinal tract, regular screening and early detection are important for people who are at average risk and have a higher risk, such as those with UC or CD. If CRC is detected at an early stage, the 5-year survival rate is 90%. Unfortunately, less than 40% of CRC are detected at an early stage[19]. Once the cancer has spread regionally and involves adjacent organs or lymph nodes, this rate drops to 40%-65%; 5-year survival is less than 10% for patients with distant metastases[20].

Many existing screening tools, invasive or not, are often debated such as FOBT, sigmoidoscopy and complete colonoscopy. New tools are in development and have to be evaluated in current practice: VC, new endoscopic technologies, DNA on faeces or proteomics with markers in serum. When used appropriately, screening for CRC can reduce disease-related morbidity and mortality[21]. Recent studies stress the fact that finding and resecting advanced adenomatous polyps, and thereby preventing cancer, is becoming a primary objective of screening programs. Several papers also show the potential of emerging new methods of screening by imaging the colon with VC[22]. The sensitivity of VC for large adenomas and CRC appears to be high, although results vary by center and there is a steep learning curve. (Table 1).

BACKGROUND OF VIRTUAL CONLONOSCOPY

VC is a new method for studying the colon. It consists in acquisition of CT, MR and PET/CT images and could elaborate them with a workstation, creating endoluminal vision as likely as traditional colonoscopy does, permitting the complete exploration of colonic lumen, and of tumoral stenosis[23]. The analysis of the differences between CT, MR and PET/CT colonography shows that these techniques present both advantages and disadvantages, such as the impossibility to perform MR in patients with pace-maker or in claustrophobic patients and the impossibility to perform CT with iodated agents in patients with renal failure or with a story of adverse reactions. The increased use of these techniques is due to the high sensitivity of last-generation CT, MR and PET/CT machine, to the increased spatial resolution, to specific softwares for digital cleaning of colon, to the introduction of high-end workstations and to the possibility of computer assisted diagnosis (CAD)[24]. So, it is desirable that the increasing spread of multidetector CT devices

Table 2 Comparison of medical image modalities' values in colonography

Modalities	Specificity	Sensitivity	Accuracy
DCBE	+	+	-
Colonoscopy	+++	++	+++
CTC	+	++	_
MRC	++	+	+
PET/CTC	++	++	+++

Legend: _- poor; + fair; +++ good; ++++ excellent.

and the future technical innovations should have the effect to increase culture and experience in various diagnostic centers about VC, making possible the spreading of VC as a screening tool (Table 2)[25].

CT COLONOGRAPHY

Current CT techniques require meticulous bowel preparation and gas insufflations prior to the examination. The procedure requires a scan time of about 25 to 30 s with new multidetector CT scanners, and sedation is not used. The advantages of CTC over CC include its safety, its ability to demonstrate the entire large bowel in almost all patients, even following incomplete endoscopy, to accurately localize lesions, and to examine the entire colon in patients with obstructing tumors. Additionally, CTC allows simultaneous preoperative tumor staging. There are few reported complications from CTC[26,27].

People with ulcerative colitis or Crohn's disease

Over the past several years, significant advances have been made in the diagnostic techniques used in the management of UC and CD. Improved radiographic imaging techniques based on CT imaging allow noninvasive means of evaluating the small bowel in patients with known or suspected CD[28]. CTC may help to distinguish between patients with acute and chronic inflammatory bowel disease (IBD). Especially extraintestinal complications, tumorous as well as pseudo-tumorous lesions can be detected with high sensitivity and specificity. CTC is clinically useful for the evaluation of CD, especially those with stenotic lesions[29,30]. Current findings also suggest that although the widespread use of VC in CD is currently not indicated because of possible false-negative findings, this technique may represent an alternative to CD in noncompliant postsurgical patients with a rigid stenosis not allowing passage of the endoscope[31].

People with polyps

Screening for colorectal polyps is a controversially discussed indication for CTC. Sensitivity and specificity range widely and decrease with decreasing polyp size. However, better results can be achieved using multidetector technology. Most frequently, the examination is well tolerated and assessed by patients to be more acceptable than CC[32]. A meta-analysis of 2610 patients, 206 of whom had large polyps, showed high per-patient average sensitivity (93%) and specificity (97%) for colonography; sensitivity and specificity decreased to 86% and 86%, respectively, when the threshold was lowered to include medium polyps. When polyps of all sizes were included, studies were too heterogeneous in sensitivity (range, 45%-97%) and specificity (range, 26%-97%) to allow meaningful meta-analysis. CTC seems sufficiently sensitive and specific in the detection of large and medium polyps[33,34].

The sensitivity of CTC was heterogeneous but improved as polyp size increased (48% for detection of polyps < 6 mm, 70% for polyps between 6 and 9 mm, and 85% for polyps > 9 mm). Characteristics of the CTC scanner, including width of collimation, type of detector, and mode of imaging, explained some of this heterogeneity. In contrast, specificity was homogenous (92% for detection of polyps < 6 mm, 93% for polyps from 6 to 9 mm, and 97% for polyps > 9 mm). CTC is highly specific, but the range of reported sensitivities is wide[35]. Patient or scanner characteristics do not fully account for this variability, but collimation, type of scanner, and mode of imaging explain some of the discrepancy. This heterogeneity raises concerns about consistency of performance and about technical variability. These issues must be resolved before CTC can be advocated for generalized screening for CRC[36].

Diagnosis and staging of colorectal cancer

Because CRC has widely varying appearances in both endoscopy and CTC, familiarity with the gamut of morphologic appearances can help improve interpretation of the results[37,38]. The addition of intravenous contrast material to CTC can aid differentiation of true colonic masses from pseudolesions such as residual stool and improves the depiction of enhancing masses that might otherwise be obscured by residual colonic fluid[39]. In contrast to staging of most other tumors, staging of CRC depends more on the depth of tumor invasion than on the size of the primary mass. The diverse appearances of colorectal cancers at two- and three-dimensional CTC include sessile, annular, ulcerated, necrotic, mucinous, invasive, and noninvasive lesions[40]. Imaging pitfalls that can simulate or obscure neoplasms are retained fecal material or fluid, incomplete distention, and advanced diverticulosis[41].

Contrast-enhanced CTC can simultaneously evaluate metastatic disease, local recurrence, and metachronous neoplasia in CRC, in recurrent CRC. Contrast-enhanced CTC has the potential to detect local recurrence, metachronous disease, and distant metastases in patients with a history of invasive CRC. Suboptimal sigmoid distention can be seen on contrast-enhanced CTC, predominantly in patients with right hemicolectomies[42]. Contrast-enhanced CTC is a promising method for detecting local recurrence, metachronous disease, and distant metastases in patients with prior invasive CRC. The technique can also serve as a useful adjunct to colonoscopy by detecting local recurrences or metachronous disease that are endoscopically obscure or by serving as a full structural colonic examination when endoscopy is incomplete[43].

MR COLONOGRAPHY

MRC has gained access into clinical routine as a means

for the assessment of the large bowel. There are widely accepted indications for MRC, especially in patients with incomplete CC. Furthermore, virtual MRC is more and more propagated as a screening tool, with advantages especially inherent to the non-invasive character of this procedure and the lack of ionizing radiation exposition. Beyond a sufficiently high diagnostic accuracy, outstanding patient acceptance is a major advantage of MRC as a diagnostic modality[44]. Precondition for establishment of MRC as a diagnostic tool in secondary prevention of CRC is not only high diagnostic accuracy but also a good acceptance amongst patients[45].

People with ulcerative colitis or Crohn's disease

The degree of the contrast enhancement of the bowel wall may be a criterion for the degree of inflammation in CD[46]. This study provides strong evidence that the combination of MRI enteroclysis and MRC is practicable and supplies additional results regarding the regions which are not seen with ileo-colonoscopy in the work-up of patients with CD[47]. The presented data indicate that 'fecal tagging MRC' is not suitable for an adequate quantification of inflammatory diseases of the large bowel. Furthermore, overall acceptance of endoscopic colonoscopy was superior to MRC[45,48]. MRC represents a promising alternative to CC for the assessment of colonic anastomoses in patients with previous colonic resection[49]. In summary, whether or not the survival of endoscopy is under debate, MRI could mark a historic turning point in gastroenterology. So, MRI hardware might interdisciplinarily be used by, e.g., radiologists and gastroen terologists[50].

People with polyps

Dark-lumen MRC has failed to detect all polyps smaller than 5 mm in diameter which are generally not clinically relevant at the moment of their detection and thus can be kept under surveillance. However, MRC as a non-invasive imaging modality is a promising alternative to CC in the detection of clinically relevant polyps larger than 5 mm in diameter[51]. The diagnostic performance of MRC was prospectively evaluated by using limited bowel preparation in patients with polyps of 10 mm or larger in diameter in a population at increased risk for CRC, with CC as the reference standard. Two hundred patients (mean age, 58 years; 128 male patients) were included; 41 patients had coexistent symptoms. At colonoscopy, 12 patients had 22 polyps of 10 mm or larger. Per-patient sensitivity was 58% (seven of 12) for observer 1, 67% (8 of 12) for observer 2, and 75% (9 of 12) for both observers combined for polyps of 10 mm or larger. Per-patient specificity was 95% (178 of 188) for observer 1, 97% (183 of 188) for observer 2, and 93% (175 of 188) for both observers combined. Per-polyp sensitivity was 55% (12 of 22) for observer 1, 50% (11 of 22) for observer 2, and 77% (17 of 22) for both observers combined. Interobserver agreement was 93% for identification of patients with lesions of 10 mm or larger. In patients at increased risk for CRC, specificity of MRC by using limited bowel preparation was high, but sensitivity was modest[52-54].

Diagnosis and staging of colorectal cancer

MRC is useful for detection of colonic pathology and assessment of proximal colon in patients with colonic cancer after incomplete colonoscopy[55-56] Wong *et al* reported 51 patients with incomplete colonoscopy who were recruited to have MRC performed. Forty-four patients had incomplete colonoscopy because of an obstructing tumor. The other seven patients had incomplete colonoscopy because of excessive bowel looping. Apart from one patient suffering from chronic obstructive airway disease with resulting nondiagnostic MRC, all other patients had MRC successfully performed. Each colon was divided into six bowel segments for analysis. All 300 segments were of diagnostic quality and were assessed by the MRC. MRC correctly identified all 44 obstructing tumors demonstrated by initial CC. Synchronous tumors in proximal colonic segments were identified in two patients by MRC. In addition, MRC identified two colonic tumors located in bowel segments inaccessible by CC because of excessive looping[57]. A meta-analysis had MRC versus colonoscopy as a diagnostic investigation for CRC. This study suggests that MRC is an imaging technique with high discrimination for cases presenting with CRC. The exact diagnostic role of MRC needs to be clarified (e.g. suitable for an elderly person with suspected CRC. Further evaluation is necessary to refine its applicability and diagnostic accuracy in comparison with other imaging methods such as CTC[58]. MRC could be useful in screening programs of patients at high risk for colon cancer. Patients with MRC-detected endoluminal lesions must undergo CC for histologic diagnosis[59].

PET/CT COLONOGRAPHY

Endoscopic and radiologic studies are frequently required in IBD to determine disease activity, extent of disease, and delineating disease type. PET and PET/CT using fluorine-18-fluoro-deoxyglucose to identify metabolically active tissues may offer a simple noninvasive alternative to conventional studies in identification and localization of active intestinal inflammation[60]. Today, PET imaging has been shown to be superior to conventional CT staging when assessing patients with CRC for local and distant metastases. PET/CT scanners provide accurately fused morphological and functional imaging within a single examination. Thus a combined whole body PET/CTC approach may serve as an attractive alternative to a multistep multimodality workup comprising CT, PET/CT, and CC[61-63].

Detection of ulcerative colitis or Crohn's disease

Over the past several years, significant advances have been made in the diagnostic techniques used in the management of UC and CD. Detection of disease activity in IBD is of crucial importance for diagnosis and management of the disease[64]. ^{18}F-fluorodeoxyglucose (18F-FDG) accumulates in malignant tissues but also at the sites of infection and inflammation and in autoimmune and granulomatous diseases by the overexpression of distinct facultative glucose transporter (GLUT) isotypes (mainly GLUT-1 and GLUT-3) and by an overproduction of glycolytic

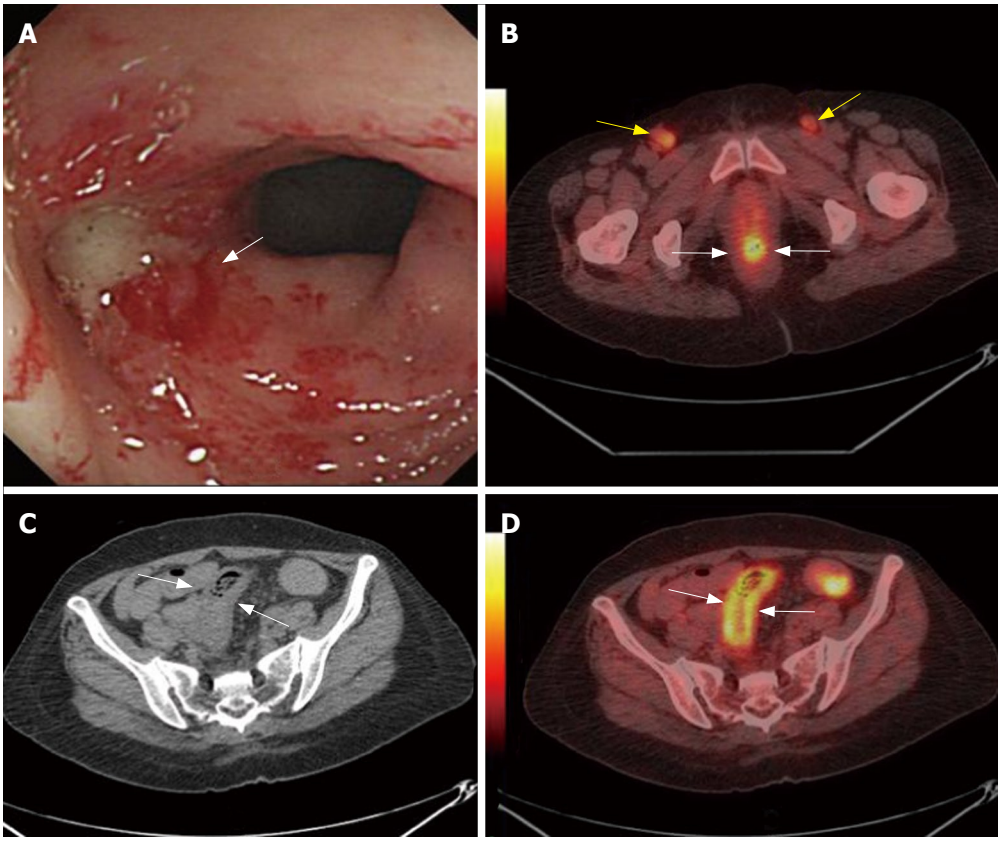


Figure 1 A 56-year-old woman was suffered from uterine cervix cancer and accepted local radiation and chemotherapy 2-mo ago. CC found enterocolitis of sigmoid colon (white arrow, **A**). PET/CT detected high metabolism metastasis lymphoid nodes at both sides of the inguina (yellow arrows, **B**) and rectum wall was high metabolism (white arrows, **B**). CT showed thickening of sigmoid colon wall (white arrows, **C**). PET/CT illustrated sigmoid colon wall was high metabolism (white arrows, **D**). Biopsy followed by PET/CT revealed metastasis lymphoid nodes at both sides of the inguina. The case illustrated the potential value of PET/CTC in radiation enterocolitis.

enzymes in cancer cells and inflammatory cells[65]. It is confirmed that PET activity correlated well with active inflammation in both UC and CD, suggesting that this may be a noninvasive method of identifying disease activity in patients with IBD[66]. Neurath *et al* suggested that FDG-PET appear to be a reliable noninvasive tool for simultaneous detection of inflamed areas in the small and large bowel of patients with CD and can be used to detect disease activity in the terminal ileum and colon of CD patients with high sensitivity and specificity[67].

PET or PET/CT in pediatric inflammatory bowel disease

Diagnosis of chronic IBD in children requires noninvasive, atraumatic diagnostic tools that depict localization and acuity of inflammation and yield only a low radiation dose. Loffler *et al* reported a retrospective analysis with histology as the standard of reference, FDG-PET showed a sensitivity/specificity/accuracy of 98%/68%/83% as compared to CC (90%/75%/82%) and US (56%/92%/75%). For the small bowel, FDG-PET was even more reliable (100%/86%/90%). Because of its high sensitivity and accuracy, FDG-PET is an excellent, noninvasive diagnostic tool for IBD. Depicting inflammation in the whole bowel, while being not traumatic, it is attractive for use especially in children. FDG-PET is especially reliable for the small bowel and can inform application of topical therapy[68,69]. However, curtailed anatomical information provided by FDG-PET often renders accurate localization of lesions difficult. PET/CT offers a better noninvasive tool for identifying and localizing active intestinal inflammation in patients with IBD. PET and PET/CT may not be able to replace conventional studies; however, it may be useful when conventional studies cannot be performed or fail to be completed[70].

PET or PET/CT in radiation enterocolitis

Radiation enteritis is a functional disorder of the large and small bowel that occurs during or after a course of radiation therapy to the abdomen, pelvis, or rectum. The large and small bowels are very sensitive to ionizing radiation. Almost all patients undergoing radiation to the abdomen, pelvis, or rectum will show signs of acute enteritis. Injuries clinically evident during the first course of radiation and up to 8 wk later are considered acute[71]. Chronic radiation enteritis may present months to years after the completion of therapy, or it may begin as acute enteritis and persist after the cessation of treatment. Only 5% to 15% of persons treated with radiation to the abdomen will develop chronic problems[72]. According to our initial experiences, FDG-PET/CT also has a potential value in management of radiation enterocolitis and other radiation complications (Figure 1).

Whole body staging and restaging of colorectal cancer by PET/CT

Staging of patients with CRC often requires multimodality, multistep imaging approach. PET/CTC is the latest technique used in CRC screening and staging, which could complete the TNM staging in one-stop examination. Kuehle et al performed a rodent polyp model to assess the feasibility of PET/CTC for the detection of colorectal masses in a rodent polyp model in an intraindividual comparison with dark-lumen MRC. Detection of small

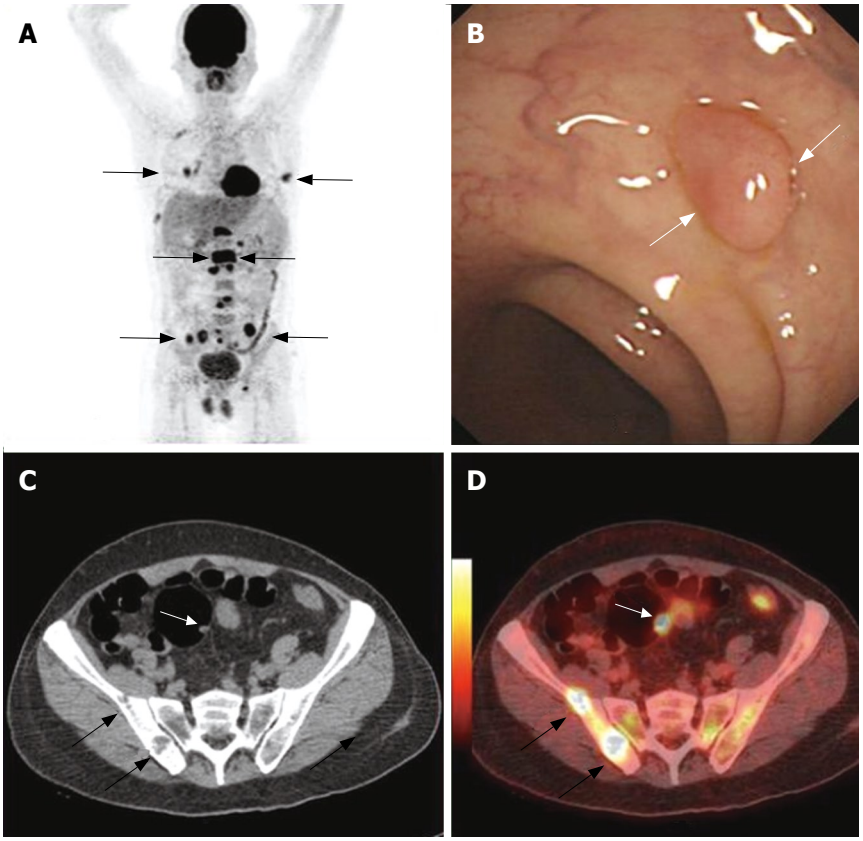


Figure 2 A 63-year-old man was detected multi metastases in whole body PET/CT scan with unknown original tumor (black arrows, **A**). CC showed a polyp at the sigmoid colon (white arrows, panel **B**). CT detected bone destruction at the right ilium (black arrow, **C**) and a polyp at the sigmoid colon (white arrow, **C**). PET/CTC localizes the high metabolism bone destruction lesions (black arrows, **D**) and high metabolism polyps at sigmoid colon (white arrow, **D**). Histopathology follow by CC revealed a inflame polyp. The case illustrated the potential value of PET/CTC in screening colorectal polyps.

tumors with PET/CTC and MRC is possible in a rodent model[73]. PET/CTC is equivalent to CT + PET for tumor staging in patients with CRC. PET/CTC in conjunction with optical colonoscopy may be a suitable concept of tumor staging for patients with CRC[74]. Kinner *et al* developed and evaluated a combined whole-body PET/CTC protocol for dedicated CRC staging in routine clinical use. The results showed that staging patients with whole-body PET/CTC is technically feasible and accurate and patients with incomplete colonoscopy or potential synchronous bowel lesions might benefit from this approach (Figure 2)[75].

Whole body PET/CT with integrated colonography is technically feasible for whole body staging in patients with CRC. PET/CT has altered management plan in 24% of patients with primary CRC in correct direction. PET/CT should be considered a part of standard workup for preoperative evaluation in a subset of patients with CRC[76]. CT and superparamagnetic iron oxide-enhanced MR imaging are more sensitive but less specific than PET in the detection of liver metastases. PET/CT can detect more patients with extrahepatic tumor than CT alone[77]. The sensitivity, specificity and overall accuracy of PET in detecting intra-abdominal extrahepatic CRC were 82%, 88% and 86%, respectively, compared with 88%, 94% and 92%, respectively, of PET/CT. For detection of extra-abdominal and/or hepatic CRC, these were 74%, 88% and 85% of PET and 95%, 100% and 99% of PET/CT. The sensitivity, specificity and overall accuracy of PET in detecting primary tumors were 80%, 69% and 75%, respectively, compared with 89%, 92% and 90%, respectively, of PET/CT. PET/CT appears to be a very promising method for distinguishing a viable tumor from fibrous changes, thereby avoiding unnecessary laparotomy[78]. This integrated protocol may be of substantial benefit in staging and restaging patients with CRC (Figure 3)[79].

CT COLONOGRAPHY *VS* MR COLONOGRAPHY

A meta-regression technique was performed to compare the diagnostic accuracy of CTC and MRC, compared with CC for patients presenting with CRC. Overall sensitivity and specificity of CTC (95%, 95% respectively) and MRC 95%, 95% respectively) in detection of CRC was similar. Meta-regression analysis showed no significant difference in the diagnostic accuracy of both modalities. Both tests showed large area under the summary receiver operating characteristic curve, with high diagnostic odds ratios. Factors that enhanced the overall accuracy of MRC were the use of intravenous contrast, faecal tagging and exclusion of low-quality studies. This meta-analysis suggested that CTC and MRC have similar diagnostic accuracy in detecting CRC. Study quality, size and intravenous/intra-luminal contrast agents affect diagnostic accuracies. For an exact comparison to be made, studies evaluating CTC, MRC and CC in the same patient cohort would be necessary[80-82].

VIRTUAL COLONOSCOPY *VS* COVENTIONAL COLONOSCOPY

CTC is reliable for detecting lesions 6 mm or larger in size. It permits evaluation of the region proximal to an occlusive

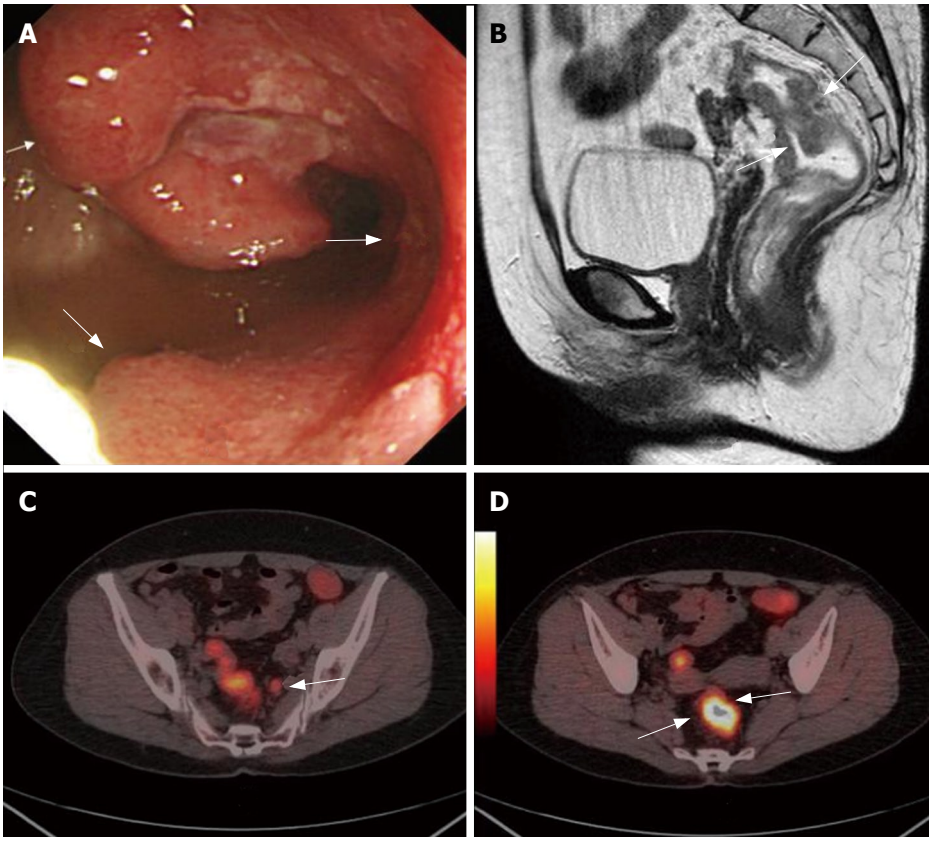


Figure 3 A 56-year-old women complained about blood stool for about 1 year. CC showed one stenotic tumoral site in the rectum (white arrows, **A**). Sagittal MRC showed thickened rectum with suspected infiltration of the surrounding tissue (white arrows, **B**). Axial PET/CTC showed a high metabolism metastasis lymphoid node (white arrow, **C**). Axial PET/CTC revealed tumor sites with elevated glucose metabolism and clear circumscription (two white arrows, **D**). PET/CT also indicated no tumorous infiltration of the adjacent tissue and no distant metastases. This was later verified by histopathology. The case illustrated the value of VC in CRC TNM staging.

growth, which is often impossible with CC[83]. CTC is a good imaging tool for the exclusion of CRC in a population unfit for or unable to complete colonoscopy or barium enema, with reasonable sensitivity and specificity for detection of CRC[84]. MRC is a promising modality with high accuracy for detecting colorectal polyps larger than 5 mm in diameter. In IBD, MRC can be used to assess disease activity, including spreading[85-86]. In detecting colon lesions, MRC achieved a diagnostic accuracy similar to CC. However, MRC is minimally invasive, with no need for sedation or analgesics during investigation. There is a lower percentage of perforation risk, and all colon segments can be evaluated due to multi-sectional imaging availability; intramural, extra-intestinal components of colonic lesions, metastasis and any additional lesions can be evaluated easily[87]. MRC is a feasible and useful method of evaluating the entire colon in patients with incomplete CC. The majority of the patients found MRC less unpleasant than CC and a majority would prefer MRC over CC as a future colon examination. MRC also appears to be less time consuming to the patients and medical personnel than CC with post-procedural monitoring[88,89]. MRC proved reliable in evaluating the majority of colonic segments inaccessible with CC. The identification of additional disease at MRC underscores the need for a second diagnostic step in the setting of incomplete CC[90].

COMPLICATIONS AND LIMITATIONS

Perforation of the colon and rectum is a rare complication of VC. Older age and underlying concomitant colonic disease were present in patients with perforation[91]. The cancer risks associated with the radiation exposure from VC are unlikely to be zero, but they are small. A best estimate for the absolute lifetime cancer risk associated with the radiation exposure using typical current scanner techniques is about 0.14% for paired VC scans for a 50-year-old, and about half that for a 70-year-old. These values probably could be reduced by factors of 5 or 10 with optimized VC protocols. In terms of the radiation exposure, the benefit-risk ratio is potentially large for VC[92].

Many differences between the studies with high sensitivity (94%)[93]. Additional obstacles for implementation in prevention of CRC may be controversial results concerning patient acceptance, the large-scale use of ionizing radiation, difficulties in detecting flat adenomas, and extracolonic findings[94]. Flat lesions and small polyps are the other two main causes for missed lesions at 8 multi-detector rows CTC[95,96]. Currently, CTC is less cost-effective than conventional endoscopy[97]. CTC and MRC present both advantages and disadvantages, such as the impossibility to perform MR in patients with pace-maker or in claustrophobic patients and the impossibility to perform CT with iodated agents in patients with renal failure or with a story of adverse reactions[98,99]. Although application of PET and PET/CT in CRC diagnosis, staging and re-staging has been widely accepted by oncologists, there are no data of PET/CTC in CRC screening. But in the high risk patients and follow up of CRC patients after treatment, the examination is still cost-effective. Radiation dose exposures to the patient who accepts this examination is another main problem which should be further studied in the near future[100,101].

COST-EFFECTIVE ISSUES

CTC is an effective screening test for colorectal neoplasia.

However, it is more expensive and generally less effective than CC. CTC can be reasonably cost-effective when its diagnostic accuracy is high, as with primary 3-dimensional technology, as if costs are about 60% of those of CC. Overall, CTC technology will need to improve its accuracy and reliability to be a cost-effective screening option[102]. CRC screening is cost-saving in Italy, irrespective of the technique applied. CTC appeared to be more cost-effective than flexible sigmoidoscopy, and it may also become a valid alternative to CC[103]. VC involves a helical CT or MR scan of the abdomen and pelvis to detect colorectal polyps and cancer. Both modalities have shown promising sensitivity in revealing larger polyps, in comparison with CC. Caution should be exercised in its clinical implementation due to significant interobserver variation and individual learning curves. A Danish study indicates that CTC can be performed cost-effectively compared to CC. CTC is recommended in preference to DCBE after incomplete CC[104].

DERIVATIVE TECHNIQUE FOR CRC SCREENING

Stool DNA testing

Stool DNA testing provides an acceptable noninvasive alternative for CRC screening that can identify early-stage CRC and adenomatous polyps in routine clinical practice[105]. Abbaszadegan[106] *et al* reported that this test could detect CRC related genetic alterations by analyzing stool DNA with a sensitivity of 64% and 20% and a specificity of 95% and 100% for long DNA and p16 respectively. An abnormal stool DNA test correlated with a colonoscopically demonstrable abnormality in 49% of cases (34 of 69). Abnormal findings, including CRC in 4% of cases (3 patients), single or multiple adenomatous polyps in 23 patients (33%), hyperplastic polyps in 3 patients (4%), and colitis in 5 patients (7%). Colonoscopy was reported as negative in 51% of patients (35 of 69), including 2 cases (3%). A non-invasive molecular stool-based DNA testing can provide a screening strategy in high-risk individuals[107].

Computer-aided detection

The latest CAD systems indicate a clinically acceptable high sensitivity and a low false-positive rate, and observer studies have demonstrated the benefits of these systems in improving radiologists' detection performance[108]. Polyps were classified as small ($\leqslant$ 5 mm), medium (6-9 mm), and large ($\geqslant$ 10 mm). A total of 118 polyps (small, 85; medium, 19; large, 14) were found in 56 patients. CAD detected 72 polyps (61%) with an average of 2.2 false-positives. Sensitivity was 51% (43/85) for small, 90% (17/19) for medium, and 86% (12/14) for large polyps. For all polyps, per-patient sensitivity was 89% (50/56) for the radiologist and 73% (41/56) for CAD. For large and medium polyps, per-patient sensitivity was 100% for the radiologist, and 96% for CAD. CAD shows high sensitivity in the detection of clinically significant polyps with acceptable false-positive rates[109] and CAD for CTC significantly increases per-patient and per-polyp detection and significantly reduces interpretation times but cannot substitute adequate training[100].

PET/MRI colonography

Predicting the future is difficult, and any attempt to do so is fundamentally flawed. But the fusion of PET with MRI could compensate for their disadvantages and therefore offers several advantages in comparison to PET or MRI alone. The combination of these two excellent diagnostic imaging modalities into a single scanner improves the diagnostic accuracy by facilitating the accurate registration of molecular aspects and metabolic alterations of the diseases with exact correlation to anatomical findings and morphological information. PET/MR imaging is to become a multimodality platform for molecular imaging and PET/MRI colonography might be a promising diagnostic modality for use in CRC screening in the decades to come due to the considerably lower radiation exposure in contrast to PET/CTC and the high soft tissue resolution of MRI in contrast to CT in the future[110-101].

CONCLUSION

With the exponential development in computer processing power, CT, MR and PET/CT colonography offer numerous advantages over more traditional methods of radiologic diagnosis, and provide essential information not only for initial diagnosis, but also for management, follow-up and detection of potential complications. Will CT, MR and PET/CT colonography replace conventional colonoscopy in the future? We do not believe so at present. However, combined with several derivative techniques on the horizon involving stool DNA testing, computer-aided detection and PET/MRI colonography, these techniques may further improve the specificity and sensitivity of imaging modalities in CRC screening and save the colonoscopy resource for the patients who need treatment.

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TOPIC HIGHLIGHT

Etienne M Sokal, *Series Editor*

Stem cells for liver tissue repair: Current knowledge and perspectives

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Abstract

Stem cells from extra- or intrahepatic sources have been recently characterized and their usefulness for the generation of hepatocyte-like lineages has been demonstrated. Therefore, they are being increasingly considered for future applications in liver cell therapy. In that field, liver cell transplantation is currently regarded as a possible alternative to whole organ transplantation, while stem cells possess theoretical advantages on hepatocytes as they display higher *in vitro* culture performances and could be used in autologous transplant procedures. However, the current research on the hepatic fate of stem cells is still facing difficulties to demonstrate the acquisition of a full mature hepatocyte phenotype, both *in vitro* and *in vivo*. Furthermore, the lack of obvious demonstration of *in vivo* hepatocyte-like cell functionality remains associated to low repopulation rates obtained after current transplantation procedures. The present review focuses on the current knowledge of the stem cell potential for liver therapy. We discuss the characteristics of the principal cell candidates and the methods to demonstrate their hepatic potential *in vitro* and *in vivo*. We finally address the question of the future clinical applications of stem cells for liver tissue repair and the technical aspects that remain to be investigated.

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Key words: Stem cells; Hepatocyte differentiation; Liver regeneration; Cell therapy

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INTRODUCTION

Orthotopic liver transplantation (OLT) is the gold standard treatment for end-stage liver failure and for numerous liver based inborn errors of metabolism. However, organ shortage remains a major limiting factor and alternative solutions are being examined in the liver therapy field. Liver cell transplantation (LCT) is emerging with heartening success[1,2], but is still limited by cell viability, modest engraftment and limited tissue availability. Increasing interest is carried to stem cells regarding the recent demonstration of their plasticity[3]. Theoretical advantages of stem cells for tissue regenerative medicine are multiple: ease of harvest, proliferation capacity, efficiency of *in vitro* transfection and potential use of autologous cells. Different types of stem cells are eligible for liver cell therapy according to their hepatic potential, for instance mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs) and adult liver stem/progenitor cells. Despite encouraging results, key pitfalls remain while using stem cells-derived hepatocyte-like cells: lack of tissue-specific functionality and, up to now, no evidence of strong liver repopulation level in animal models. Moreover, the demonstration of the acquired hepatocyte-like phenotype is impaired by technical restraints. To reach clinical application, stem cell therapy requires further development to become competitive regarding LCT. Here, we present the current knowledge on the use of stem cells for hepatic tissue engineering and describe the future orientations in the field.

IDENTIFICATION OF THE STEM CELL CANDIDATES

Mesenchymal stem cells

Firstly described in 1970 from bone marrow (BM) isolates[4], these cells can currently be obtained from various tissue sources as BM, cord blood (CB), adipose tissue, umbilical cord blood (UCB). Their *in vivo* function

Table 1 Representative studies investigating *in vitro* and/or *in vivo* hepatocyte differentiation of MSCs

Cell source	*In vitro* differentiation	*In vivo* differentiation	References
Human BM&UCB immunoselection	Cocktail: EGF-bFGF (2 d), HGF-bFGF-nicot (7 d), OSM-dexa-ITS Characterization: RT-PCR (8), ICC (2), urea production, LDL uptake, PROD	ND	Lee *et al*[8]
Human UCB	ND	Host: preimmune fetal sheep Injury: - Cell characterization: Alb^+, $HepPar1^+$ Fusion: microdissection + PCR: - Repopulation rate: > 20%	Kögler *et al*[16]
Human adipose tissue	Cocktail: DMSO-HGF-OSM Characterization: RT-PCR (4), ICC (1), urea production, LDL uptake	Host: NOD/SCID mice Injury: CCl_4 TRP: 1.10^6 cells, tail vein Cell characterization: Alb^+/CM-Dil^+ Repopulation rate: up to 0.13% Alb^+ at 10 d	Seo *et al*[22]
Human BM MSCs, $CD34^+$ cells, non MSCs/$CD34^-$ cells	ND	Host: WT rats (+CsA) Injury: AA TRP: 1.10^6 cells, liver Cell characterization: Alb^+, $CK18^+$, $CK19^+$, αFP^+, $AGPR^+$, serum Alb^+ Fusion: FISH hum Y/rat chr 12: -	Sato *et al*[21]
Human BM	Cocktail: 5'aza (1 d) - EGF-EGF (14 d) Characterization: RT-PCR (11), ICC (4), WB (4), urea production, PAS, reporter gene construct for PEPCK activity	Host: Pfp/$Rag2^{-/-}$ mice Injury: propanolol, partial hepatect TRP: 1.10^6 cells, liver Cell characterization: Alb^+, $HepPar\text{-}1^+$, $PEPCK^+$, $CX32^+$, PAS^+ Fusion: FISH hum alu/mouse satellite: -	Aurich *et al*[10]
Human BM	Cocktail: HGF-FGF4-ITS-nicot (7 d), OSM-dexa-ITS (15 d) Characterization: RT-PCR (17), ICC (8), flow cytometry, urea&Alb production, PAS, G6Pase activity, neoglucogenesis	Host: SCID mice Injury: partial hepatect or retrorsine/AA TRP: 1.10^6 cells, spleen or liver Cell characterization: Alb^+, αFP^+, $CK18^+$, $fibronectin^+$, $vimentin^+$, hum alu^+	Lysy *et al*[106]

Alb: Albumin; AA: Allyl alcohol; αFP: Alpha-fetoprotein; CCl_4: Carbon tetrachloride; CB: Cord blood; CK: Cytokeratin; CsA: Ciclosporin A; d: Days; dexa: Dexamethasone; FGF: Fibroblastic growth factor; G6Pase: Glucose-6-phosphatase; hepatect: Hepatectomy; HGF: Hepatocyte growth factor; hum: Human; ITS: Insulin-transferrin-selenium; nicot: Nicotinamide; OSM: Oncostatin M; PAS: Periodic acid-schiff; PEPCK: Phosphoenolpyruvate carboxykinase; PROD: Pentoxyresorufin-O-dealkylase; WT: Wild-type; chr: Chromosome; FISH: Fluorescent *in situ* hybridization; ICC: Immunocytochemistry; ND: Not documented; RT-PCR: Reverse transcription-polymerase chain reaction; TRP: Transplantation; WB: Western blot. Amounts of analyzed markers are provided between brackets.

and behavior remain largely unknown[5]. MSCs have the characteristics to be highly proliferative *in vitro*, easily transfectable, resistant to cryopreservation[6] and to display a large *in vitro* and *in vivo* differentiation potential[7]. The first description of their hepatic potential was provided by Lee *et al*[8] and confirmed by other studies[9-26] (Table 1). While some studies evidenced the *in vivo* potential of MSCs[10,21], this was recently questioned by other authors who did not detect engrafting cells after syngeneic transplantation[20]. However, experimental protocols were quite different rendering comparison hazardous. A key advantage of MSCs is their immunological properties making them lesser immunogenic and possibly able to induce tolerance as highlighted by promising *in vitro* studies and clinical trials[27-32].

Hematopoietic stem cells

These cells constitute the paradigm of stem cells: cells capable of self-renewing, proliferation and production of a progeny of committed cell lineages, permitting the regeneration of the tissue, even after transplantation. These can be isolated from BM, UCB and peripheral blood[33] making them highly available while they are still difficult to expand *in vitro*[34,35]. In this cell population, cell identity and phenotype are mainly determined by a selection procedure whose criteria remain debated[36]. HSCs have recently aroused much enthusiasm in the hepatology field after the demonstration of their hepatic differentiation potential as described in Table 2. A majority of studies used mononuclear cell preparations[37-63], which hamper the identification of the cells involved in the differentiation process. For that reason, we included in Table 2 only studies using defined subpopulations[64-80]. However, HSCs' liver potential is increasingly debated[40,65,81-84] and the possibility of cell fusion as an explanation of 'plasticity' make these cells less considerable for a clinical use[85].

Adult liver stem cells

As organ shortage is limiting the availability of this cell population, the conditions for their use in cell therapy are

Table 2 Representative studies investigating *in vitro* and/or *in vivo* hepatocyte differentiation of HSCs

Cell source	*In vitro* differentiation	*In vivo* differentiation	References
Mouse BM	ND	Host: irradiated FAH$^{-/-}$ mice Injury: NTBC withdrawal TRP: 10 to 1000 cells; retroorbital plexus Cell characterization: β-gal$^+$, Alb$^+$, DPPIV$^+$, e-cadherin$^+$, FISH Y$^+$, liver function, FAH activity Repopulation rate: 30%-50% βgal$^+$ cells	Lagasse *et al*[73]
Mouse BM	ND	Host: irradiated CD45.1$^+$ mice Injury: hepatect or anti-Fas Ab treatment TRP: 10^6 cells; vein Cell characterization: CK8$^+$, CK18$^+$, CK19$^+$, CD45.2$^+$, FISH Y$^+$, PCR Bcl-2$^+$ Repopulation rate: up to 0.05%-0.8% with anti-Fas Ab treatment	Mallet *et al*[74]
Human UCB&BM	ND	Host: irradiated NOD/SCID mice ± HGF Injury: CCl_4 TRP: 2000 to 10^5 cells; vein Cell characterization: Alb$^+$, CD45$^-$, human alu$^+$, CK19$^+$, Alb mRNA$^+$, serum Alb$^+$ Repopulation rate: < 1%	Wang *et al*[77]
Human UCB&PB	ND	Host: irradiated NOD/SCID mice Injury: CCl_4 TRP: 2 to 6 × 10^5 CD34$^+$ cells; tail vein Cell characterization: CD45$^+$, Alb$^+$, α1-AT$^+$, hum DNA$^+$, hum Alb mRNA$^+$&prot$^+$ Repopulation rate: ⩽ 0.01% hum Alb mRNA in mice *vs* human liver	Kollet *et al*[70]
Mouse BM	Cocktail: co-culture with damaged liver tissue Characterization: RT-PCR (8), ICC (10), FISH Alb mRNA	Host: mice ± irradiation Injury: CCl_4 TRP: 10^5 cells; tail vein Cell characterization: Alb$^+$, FISH Y$^+$, E-cadh$^+$, PKH26$^+$, serum liver function Repopulation rate: up to 7.6% at 2 d Fusion: FISH X-Y: -	Jang *et al*[69]
Mouse BM	ND	Host: mice ± irradiation ± rhG-CSF Injury: partial hepatect or CCl_4 TRP: 5 × 10^3 selected cells or 5 × 10^5 MNCs; spleen Cell characterization: no co-eGFP/DPPIV staining, PCR eGFP$^-$ Repopulation rate: 0/6.9 × 10^7 host hepatocytes	Cantz *et al*[65]

α1-AT: Alpha-1-antitrypsin; Ab: Antibody, Alb: Albumin; β-gal: β-galactosidase; BM: Bone marrow; CCl_4: Carbon tetrachloride; CK: Cytokeratin; d: Days; dexa: Dexamethasone; DPPIV: Dipeptidyl peptidase IV; eGFP: Enhanced green fluorescent protein; FAH: Fumarylacetoacetate hydrolase; hepatect: Hepatectomy; HGF: Hepatocyte growth factor; hum: Human; MNCs: Mononuclear cells; ND: Not documented; NTBC: 2-2nitro-4-trifluoromethylbezoyle-1,3-cyclohexanedione; PB: Peripheral blood; UCB: Umbilical cord blood; E-cadh: E-cadherin; rhG-CSF: Recombinant human Granulocyte-Colony Stimulating Factor; TRP: Transplantation. Amounts of analyzed markers are provided between brackets.

governed by two main characteristics, a high proliferation rate and/or a robust cell banking capacity. Regarding their promising hepatocyte-like functionality, some cell compartments could be promptly considered for toxicological assays. Even if identity and *in vivo* function of this cell population are currently under controversy, four main types of hepatic progenitors are described: oval cells, small hepatocytes, liver epithelial cells and mesenchymal-like cells. Oval cells are generated from the biliary tree in response to hepatic injury. They display a bipotent differentiation potential (hepatic and biliary cells) and can be expanded *in vitro*. There is no consensus on whether these cells are originating in BM or not[48,86]. However, similar cell types were recently isolated from healthy liver[87]. The first description of small hepatocytes isolation was made by Mitaka *et al*[88] from a non-parenchymal fraction after centrifugation of isolated liver cells. These cells are smaller than hepatocytes, possess an *in vitro* proliferation capacity[89] and can differentiate into mature hepatocytes *in vitro*[90]. Liver epithelial cells is a population firstly described by Tsao *et al*[91] and more recently in healthy adult human liver[92]. Although different from oval cells, these cells are bipotential and able to differentiate into hepatocyte-like cells *in vivo*[92]. A mesenchymal-like cell population has also been isolated from adult human liver[93,94]. This population depicts high

level of proliferation and, as MSCs, possesses a broad differentiation potential. How these cells share MSCs' immunological properties has to be determined. Other cell types were also isolated from manipulated livers[95], but the possibility of culture artefacts[96,97] should always be considered before describing new 'liver progenitor cells'.

Other cell types

Embryonic stem cells constitute, at present, the best *in vitro* model for hepatocyte differentiation. However, ethical restrictions[98] and the possibility of malignancies development[99] mainly limit their use in the clinical setting[100,101]. Other more committed cell lineages as monocytes[102,103] or fibroblasts[104] were evaluated for hepatic differentiation. In this context, recent data about nuclear reprogramming[105] provided possible explanation of the differentiation potential of cells thought to be restricted to a cell lineage.

IN VITRO STUDY AS ARGUMENT FOR HEPATOCYTE COMMITMENT

Up to now, studies illustrating *in vitro* hepatic features of stem cells-derived hepatocyte-like cells were essentially confined at the phenotypic rather than the functional level. Acquisition of specific markers is a tool for evidencing a cell commitment while hepatocyte-like functionality is required to consider a cell for therapy.

Hepatocyte-like phenotype

While a morphological change is common after *in vitro* cell conditioning, the morphology is rarely similar to mature hepatocytes and authors should rather talk about a 'hepatocyte-like morphology' in differentiating cells, more especially as ultrastructural data are mostly not provided. How the morphological changes are evocative of the acquisition of an epithelial phenotype is questionable as similar morphological changes can be obtained when incubating hBM-MSCs with a classical differentiation medium or with a control medium[106]. Using specific protein and mRNA marker expression for the description of a hepatocyte-like phenotype could also be questioned as many studies were based on restricted panel of markers and as some authors described the presence of hepatocyte-specific markers in undifferentiated cells[8,14,16,22,82,107,108]. The broadest amount of markers for phenotypic characterization should be used. In this manner, we observed an incomplete and erratic acquisition of hepatocyte-specific features in hBM-MSCs[106], cf Table 1. Moreover, these cells partially maintained the expression of native mesenchymal markers, suggesting a chimerical phenotype. This phenotype could be the result of the artificial *in vitro* differentiation process.

Hepatocyte-like functionality

The functional characterization of stem cells-derived hepatocyte-like cells is, up to now, principally achieved by commercially available assays (ELISA, colorimetric tests, PROD or EROD assays) whose handling are often poorly reproducible and quite difficult to interpret, or by other tests (as lipoprotein uptake) with low specificity. As clearly stated by Hengstler *et al*[109] in a recent report, there is a crucial need for standardized and normalized techniques for characterizing hepatocyte-like functionality in differentiating cells. Regarding clinical application, one should provide the evidence of a specific functional activity after differentiation before considering stem cells for cell therapy.

IN VIVO STUDY AS PROOF OF HEPATOCYTE DIFFERENTIATION POTENTIAL

Direct detection of differentiated donor cells in situ

Most studies about stem cells *in vivo* hepatic repopulation and differentiation are performed in a xenogeneic setting (e.g. human cells transplanted into immunocompromised rodents). The impact of cell-to-cell interactions in an interspecies microenvironment on cell adhesion and differentiation[110] is poorly documented. Moreover, this model does not reflect clearly the clinical reality and data must be carefully interpreted. In the same way, a drawback of allogeneic cell transplantation in rodents is that stem cell behavior was described to be quite different between species making extrapolation to human perilous[111]. Besides a huge diversity in the studies design, almost all *in vivo* models are based on the analysis of a repopulation potential after cell infusion in a diseased liver. Putative mechanisms of hepatic repopulation and tools designed to enhance it are described in Figure 1. It arises from the literature that hepatocyte differentiation from extrahepatic stem cells does not occur (or at very low level) in a non-injury model, and that the injury has to be strong enough and give advantage to the donor cells to seed and proliferate into the liver parenchyma. This was elegantly suggested by Stadtfeld *et al*[83] who demonstrated in a transgenic mouse model the minor role of the hematopoietic compartment in the adult liver ontogenetic development. Drug-induced liver injury has to be considered only as a tool for studying *in vivo* hepatocyte differentiation potential and no consensual protocol exists to date. Many questions are still opened: type of liver injury, time dependency of liver stimulus (acute *vs* chronic liver failure), use of drugs blocking host cell regeneration, relevance of injury after cell transplantation (compatibility with donor cell viability), *etc*. Globally, liver repopulation after stem cell transplantation remains limited. It is noteworthy that these data were obtained after single cell injection while for clinical purpose one would consider serial injections. Engraftment level has to be determined in this situation. By comparison, LCT can potentially reach higher repopulation levels[112] and, for that reason, is currently more valuable for clinical purpose. Conversely, some authors reported amazing repopulation levels with HSCs[73] or with UCB stem cells[16], but in both cases animal models were highly permissive and not representative of common cell transplant procedures. Methods for evaluation of cell engraftment are multiple and deal each with proper pitfalls[113]. For example, fluorescent in situ hybridization for detecting Y chromosome in a sex-mismatched transplantation

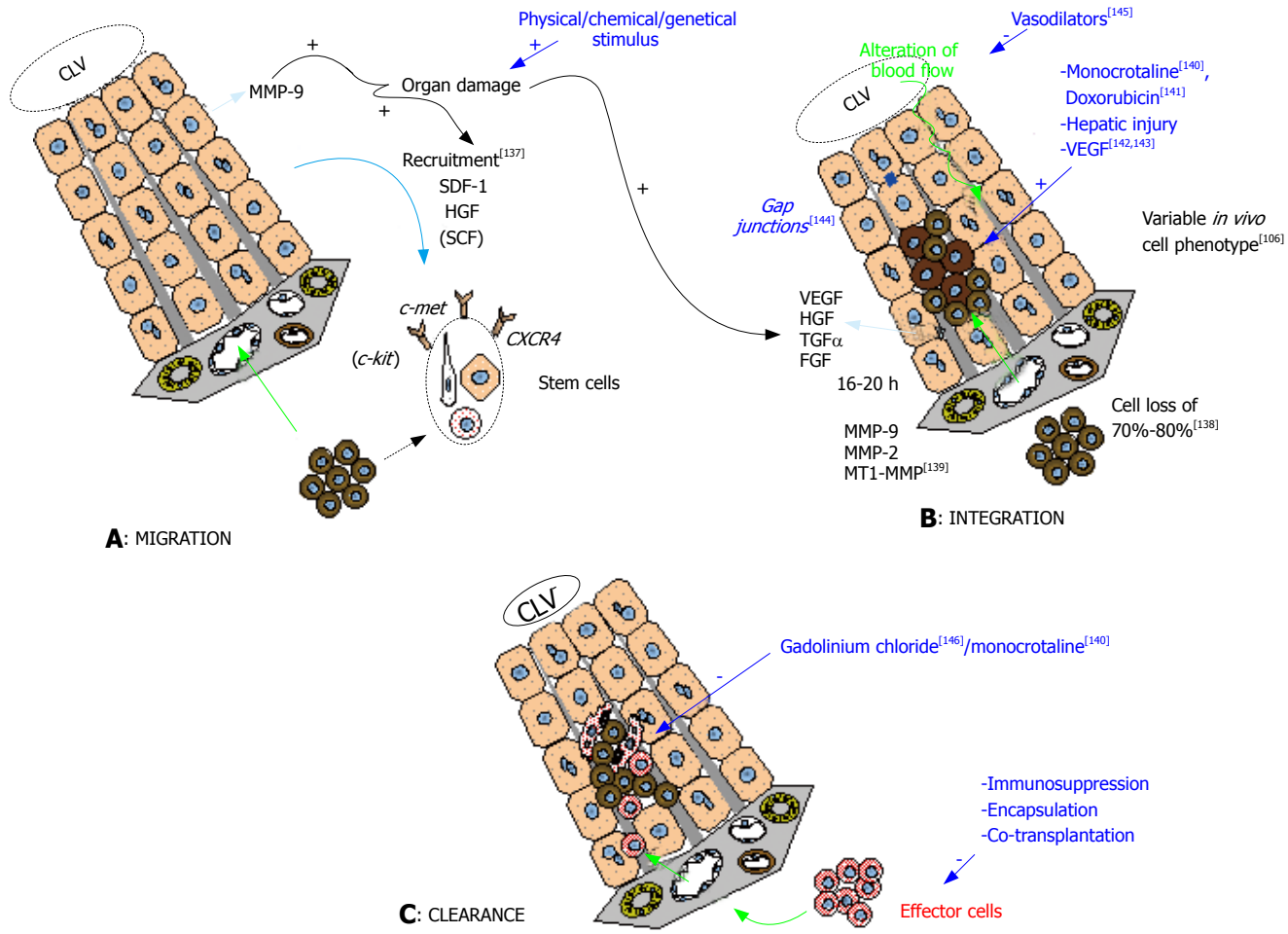


Figure 1 Mechanisms of liver repopulation after stem cell transplantation. **A**: Stem cell migration into the liver parenchyma after spleen infusion is guided mainly by the splenic and the portal vein flow. Homing of the cells is tightly regulated by chemoattractive agents (as SDF-1, HGF and SCF) secreted by liver cells under liver damage conditions[136], and acting respectively through the interaction with CXCR4, c-met and c-kit receptors of stem cells. MMP-9, expressed by host hepatocytes after injury, is implicated in the homing process through its action on the extracellular matrix. This process can be triggered by various types of liver injury; **B**: While reaching the portal vein, 70% to 80% of the infused cells are lost and phagocytosis by macrophages/phagocytes can be observed in the portal areas[137]. After 16-20 h post-infusion, the remaining cells enter the liver sinusoid after having crossed over the endothelial barrier rendered permeable by a local secretion of VEGF[137], and by the combined action of various MMPs[138]. The permeabilization of the endothelium by specific drugs owning toxicity against endothelial cells (as monocrotaline[139] or doxorubicin[140]) or acting on the permeation (VEGF[141,142]) was described to enhance the repopulation rate of the infused cells. The penetration of the parenchyma is facilitated by the action of MMPs and the disruption of gap junctions[143]. The cell implantation creates an alteration of venous blood flow which can hamper the progression of the cells inside the parenchyma but can be alleviated by the use of vasodilators[144]. Globally, inducing liver injury facilitates cell integration by remodeling the liver architecture and by creating a local microenvironment favorable for the proliferation of the transplanted cells. This is mediated by the local secretion of cytokines/growth factors (HGF, FGF, TGFα). Cell implantation implicates the reformation of gap junctions that can be observed 3 to 7 d after transplantation. The implanted cells display variable cell phenotype parallel to the level of their hepatocyte differentiation; **C**: After integration, some cells will be cleared from the hepatic parenchyma. Dead cells lacking implantation stimulus or cell-to-cell interactions will be phagocytes by Kupffer cells. This can be inhibited by the use of gadolinium chloride[145] or by monocrotaline[139] which was demonstrated in animal models to improve the kinetics of cell implantation. Other cells will be rejected by the host immunological system (effector cells) and tools to alleviate this rejection are multiple (immunosuppression protocols, encapsulation of the infused cells, co-transplantation with cells owning immunosuppressive properties). FGF: Fibroblastic growth factor; HGF: Hepatocyte growth factor; MMP: Matrix metalloproteinase; SCF: Stem cell factor; SDF: Stromal derived factor; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor.

can be difficult to interpret and, at low positivity levels, could be hampered by female microchimerism occurring after several litters[114]. The evaluation of *in vivo* cell differentiation is prominently performed by objectifying protein markers expression whereas immunoassays are often technically limited by antibodies affinity or specificity. For instance, the demonstration of *in vivo* expression of HepPar-1 (OCHE15 clone) should be questioned because of the antibody's cross-reactivity between species. For that reason, the use of combined techniques is recommended for the identification of *in vivo* differentiating cells, as staining assays performed on serial sections and association of various techniques (ex: *in situ* hybridization, immunostaining, cell tracking with fluorescent molecules, flow cytometry, RT-PCR, cellular magnetic resonance) (Figure 2).

Demonstration of donor cell functionality through their metabolic activity

The gold-standard for evaluating *in vivo* differentiation is the detection of cell functionality. To this end, some authors reported albumin secretion in transplanted mice serum. Other reports on '*in vivo* functionality' are argued on basis of the reduction of mortality rate, improvement of liver regeneration or of liver fibrosis[26,59,81,115-118]. It has to be determined how these findings are the fact of indirect

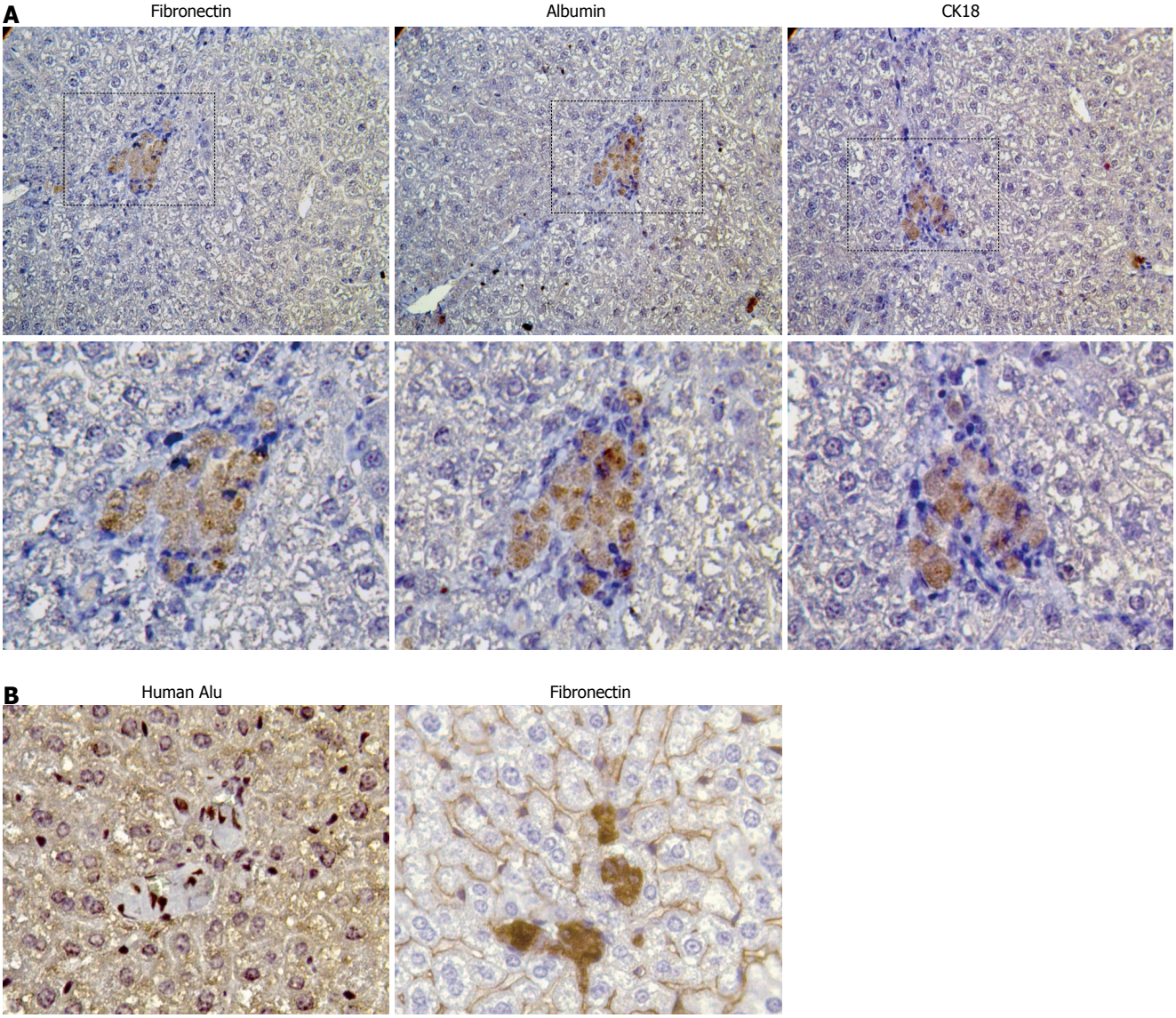


Figure 2 Analysis of liver engraftment of transplanted stem cells. Human BM-MSCs were transplanted into the liver of partially hepatectomized SCID mice and livers were screened after one month for analysis. **A**: Staining on serial sections for fibronectin, albumin and CK18 by immunohistochemistry revealed a co-expression of mesodermal and hepatocyte markers and confirmed the chimerical phenotype of hepatocyte differentiating MSCs (400 ×); **B**: The use of combined techniques for revealing the expression of human alu probe by *in situ* hybridization and of fibronectin by immunohistochemistry in a cell cluster (800 ×).

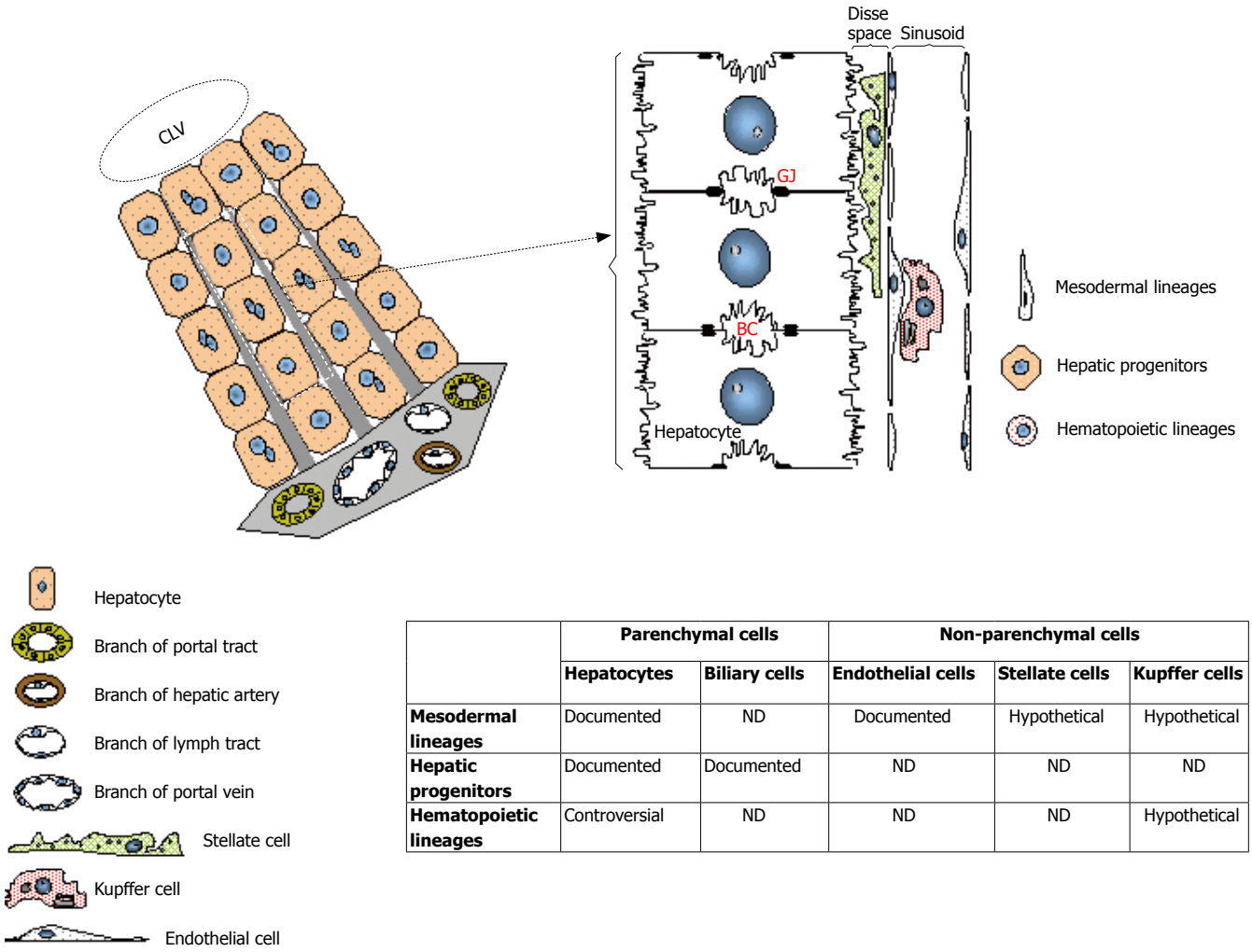
cell-to-cell interactions or of direct cell functionality. However, Lagasse *et al*[73] historically demonstrated the improvement of tyrosinemia disease in mouse after cell infusion whereas the selective pressure in this model is high and elicits fusion events.

Implication of cell fusion in the differentiation process

The fusion process implies that a cell inserts its genetic content into another cell to form a resulting unit that acquire the 'host' phenotype. The resultant product creates a heterokaryon in which the nuclei do not always fuse. The concept of fusion has emerged after experiments on co-culture of BM cells with ESCs[119,120]. Fusion between hematopoietic cells and hepatocytes has been demonstrated[43,121-125] and invalidated[69,126,127]. This is explained by the conceptual diversity of these studies and perhaps the complexity of the process itself. For example, fusion events can be acted by HSCs[128] or require homing of these cells and implication of progeny, as highlighted by studies showing fusion between myelomonocytic lineages and hepatocytes[122,125]. Interestingly, studies providing strong data about fusion used the $FAH^{-/-}$ model which produces mitotic and chromosomal abnormalities[129] that could strengthen the fusion process. It appears that selective pressure is necessary to induce relevant fusion processes which happen rarely in a non-injury model. How the cell fusion and plasticity phenomenon are parts of the same process or vary in importance according to the population used (stem cells versus committed cells[130]), has to be explored by tracing the donor cells in their route and studying signalling pathways. Interestingly, fusion events have, to date, never been described with MSCs[10,21].

FROM BENCH TO CLINICAL SIDE

Literature concerning the use of stem cells for clinical



	Parenchymal cells		Non-parenchymal cells		
	Hepatocytes	**Biliary cells**	**Endothelial cells**	**Stellate cells**	**Kupffer cells**
Mesodermal lineages	Documented	ND	Documented	Hypothetical	Hypothetical
Hepatic progenitors	Documented	Documented	ND	ND	ND
Hematopoietic lineages	Controversial	ND	ND	ND	Hypothetical

Figure 3 Involvement of stem cells in the regeneration of the liver parenchymal and non-parenchymal cell lineages. Schema shows the liver architecture and details the topography of the cell populations. In the table are resumed the cell fractions that could potentially be the targets of differentiation after transplantation of stem cells. Some of these differentiation potentials were documented with stem cells while other were contradicted and were thus exposed as controversial. Other differentiation pathways are purely hypothetical based on the embryonic origin of the stem cells. BC: Bile canalicule; CLV: Centrolobular vein; GJ: Gap junctions.

therapy is unceasingly growing in many fields (including hematology, cardiology, neurology and orthopedics)[131]. However, to date, there is no clear documentation about long-term safety of such procedure[132,133]. Multi-center consensual state-of-the-art about several clinical aspects is mandatory. First is the election of the appropriate clinical indication. In the hepatology field, liver-based inborn errors of metabolism are the most attractive candidate because of the possibility for elective treatment and, in some cases, the absence of global liver function impairment making technical procedures more feasible. The lack of liver injury may limit stem cells engraftment and differentiation. However, stem cells therapy remains relevant since small increases of the deficient enzyme activity can improve patients' quality of life. In that case, the acquisition of a specific enzyme activity has to be demonstrated *in vitro* before cell transplantation. Liver cirrhosis/fibrosis has been considered: besides encouraging results, there is, to date, no evidence about a direct or a mediated cell involvement, nor about how the fibrosis could hamper cell implantation[116]. Before considering acute liver failure for stem cell therapy, it would be necessary to achieve a performing banking of functional hepatocyte-like cells. Globally, the possibility of repeated cell infusions could render cell therapy advantageous over OLT in patients whose disorder is not life-threatening (ex: Criggler-Najar patients). In this condition, whether stem cells could have an advantage on liver cells according to their proliferative capacity needs further assessment. Other points requiring consensus is the technique and route of cell delivery (surgically placed catheter *vs* transcutaneous injection), the amount of delivered cells and timing of infusions, and the protocol of immunosuppression.

CONCLUSION

The first challenge for considering stem cells for therapy is the proof of their safety at long term by *in vitro* and *in vivo* assays. The other major goal is the determination of their *in vivo* functionality in small animal models combining a metabolic disorder allowing objective screening of cell function and a permissive immunological context (SCID or knock-out mice, immunosuppression therapy in allotransplantation). Large animal models would provide

definitive arguments about safety and functionality and allow an ideal setting for designing immunosuppression protocol and cell injection program. These models will be useful to attest the place of stem cells in liver cell therapy by comparison with LCT. Further research is also mandatory to examine the involvement of stem cells in the reconstitution of the non-parenchymal cell compartment after transplantation. Indeed, some cell populations have the potential to differentiate into endothelial cells[93,134,135] or could differentiate into mesodermal lineages similar to their phenotype (as hepatic stellate cells or fibroblasts) and as such interfere with the liver tissue architecture (Figure 3). Regarding the promising results obtained using MSCs as third-party for HSCs transplantation[29,31] or in immunological pathologies[30], the immunomodulatory role of these cells as adjuvant in LCT or in OLT should be determined.

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BASIC RESEARCH

Berberine reverses free-fatty-acid-induced insulin resistance in 3T3-L1 adipocytes through targeting IKKβ

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Abstract

AIM: To investigate the effects and molecular mechanisms of berberine on improving insulin resistance induced by free fatty acids (FFAs) in 3T3-L1 adipocytes.

METHODS: The model of insulin resistance in 3T3-L1 adipocytes was established by adding palmic acid (0.5 mmol/L) to the culture medium. Berberine treatment was performed at the same time. Glucose uptake rate was determined by the 2-deoxy-[^{3}H]-D-glucose method. The levels of IkB kinase beta (IKKβ) Ser^{181} phosphorylation, insulin receptor substrate-1(IRS-1) Ser^{307} phosphorylation, expression of IKKβ, IRS-1, nuclear transcription factor kappaB p65 (NF-κB p65), phosphatidylinositol-3-kinase p85 (PI-3K p85) and glucose transporter 4 (GLUT4) proteins were detected by Western blotting. The distribution of NF-κB p65 proteins inside the adipocytes was observed through confocal laser scanning microscopy (CLSM).

RESULTS: After the intervention of palmic acid for 24 h, the insulin-stimulated glucose transport in 3T3-L1 adipocytes was inhibited by 67%. Meanwhile, the expression of IRS-1 and PI-3K p85 protein was reduced, while the levels of IKKβ Ser^{181} and IRS-1 Ser^{307} phosphorylation, and nuclear translocation of NF-κB p65 protein were increased. However, the above indexes, which indicated the existence of insulin resistance, were reversed by berberine although the expression of GLUT4, IKKβ and total NF-κB p65 protein were not changed during this study.

CONCLUSION: Insulin resistance induced by FFAs in 3T3-L1 adipocytes can be improved by berberine. Berberine reversed free-fatty-acid-induced insulin resistance in 3T3-L1 adipocytes through targeting IKKβ.

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Key words: Berberine; Insulin resistance; IkB kinase beta; Free fatty acid

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INTRODUCTION

Berberine is an isoquinoline alkaloid extracted from *Rhizoma Coptidis*. It was initially used as a heat-clearing and detoxicating agent and an anti-inflammatory drug in clinical practice. Recently, it has been reported that activating the AMP-activated protein kinase (AMPK) pathway is the underlying mechanism for berberine improving insulin resistance, lowering blood sugar, and correcting lipid metabolism disorders[1-4]. A question will be asked if the anti-inflammatory effect of berberine is related to the effect of insulin-resistance improvement. It has been reported that salicylic acid, a traditional anti-inflammatory agent, reverses obesity- and diet-induced insulin resistance by inhibiting IkB kinase β (IKKβ)[5,6]. This has provided us with a new approach of studying the effect of berberine: is IKKβ a potential target for berberine for improving insulin resistance and relieving inflammation? This study established a model of insulin resistance induced by free fatty acids (FFAs) in 3T3-L1 adipocytes in order to observe the effects of berberine on the expression of inflammation molecules such as IKKβ, nuclear transcription factor kappaB p65 (NF-κB p65)

and insulin-signal-transduction related molecules such as insulin receptor substrate-1 (IRS-1), phosphatidylinositol-3-kinase (PI-3K) and glucose transporter 4(GLUT4), with an attempt to confirm that IKKβ is the target for berberine on relieving inflammation and improving insulin resistance.

MATERIALS AND METHODS

Drugs and reagents

Berberine (B3251), palmitic acid (PA, P0500), sodium acetylsalicylic acid (As, A5376) and fatty-acid-free bovine serum albumin (FAF BSA, A8806) were all purchased from Sigma (St Louis, MO, USA).

Cell culture and differentiation

3T3-L1 preadipocytes (obtained from Cell Center of the Institute of Basic Medicines, Chinese Academy of Medical Sciences, China) were grown to confluence in Dulbecco's minimal essential medium (DMEM; obtained from Gibco Invitrogen, USA) containing 25 mmol/L glucose and 10% fetal bovine serum (FBS; obtained from Gibco Invitrogen) at 37℃ in a humidified atmosphere containing 5% CO_2. Two days after confluence, cells were placed in DMEM containing 25 mmol/L glucose, 0.5 mmol/L isobutylmethylxanthine (15879), 1 μmol/L dexamethasone (D4902), 10 μg/mL insulin (I-6634) (all purchased from Sigma), and 10% FBS for 2 d, and then for 2 d in DMEM that contained 25 mmol/L glucose, 10 μg/mL insulin, and 10% FBS. Thereafter, cells were maintained in and re-fed every 2 d with DMEM, 25 mmol/L glucose, and 10% FBS until used in experiments 8-12 d after the start , when between 90 and 95% of the cells exhibited an adipocyte phenotype[7].

Establishment of insulin-resistance model and grouping

Fully differentiated 3T3-L1 adipocytes were cultured for 12 h in serum-free DMEM with 0.2% BSA. Then they were cultured for 24 h in DMEM containing 0.5 mmol/L PA and 1% BSA (model group), or for 24 and 48 h in DMEM containing 0.5 mmol/L PA, 10 μmol/L berberine, 1% BSA (high-dose berberine group, BH24, BH48 group), or in DMEM containing 0.5 mmol/L PA, 1 μmol/L berberine, 1% BSA for 24 h and 48 h（low-dose berberine group, or BL24, BL48 groups), or cultured in DMEM containing 0.5 mmol/L PA, 5 mmol/L As, 1% BSA for 24 h and 48 h (As group, or As24, As48 group); or in DMEM containing 1% BSA for 24 h (normal group).

Determination of glucose uptake rate

3T3-L1 preadipocytes (5×10^5/well) were differentiated to adipocytes in a 24-well plate. After serum-starvation in 0.2% BSA DMEM overnight, the cells were incubated in 0.2% BSA DMEM containing 0.5 mmol/L PA or (and) 1 μmol/L, 10 μmol/ L Ber or 5 mmol/L aspirin for 24 or 48 h. Then, the cells were incubated in 1 mL Krebs/Ringer phosphate (KRP)/HEPES (131.2 mmol/L NaCl, 4.71 mmol/L KCl, 2.47 mmol/L $CaCl_2$, 1.24 mmol/L $MgSO_4$, 2.48 mmol/L Na_3PO_4, 10 mmol/L HEPES, pH 7.4) with or without 100 nmol/L insulin for 30 min at 37℃, after being washed three times in KRP/HEPES buffer. Next, the cells were incubated in 1 mL KRP/HEPES containing 0.5 μCi/mL 2-deoxy-D-[^{3}H] glucose (purchased from Beijing Yuanzi Hi-Tech, China) for 10 min at 37℃. Finally, the cells were washed three times in ice-cold PBS and solubilized in 1 mL 0.1 mol/L NaOH for 2 h. Radioactivity was determined by liquid scintillation spectrometry. Non-specific deoxyglucose uptake was measured in the presence of 20 μmol/L cytochalasin B (Sigma), and specific glucose uptake was detected from the subtracted total uptake. Three replicate wells were set up and each experiment was repeated three times[8]. Additionally, a Cell Counting Kit-8 (CCK-8) was employed for the monitoring of the number and viability of the cells[9].

Western blot analysis

3T3-L1 preadipocytes were grown and differentiated into adipocytes in 35-mm culture dishes. After serum-starvation in 0.2% BSA DMEM overnight, the cells were incubated in 0.2% BSA DMEM containing 0.5 mmol/L PA or (and) 1 μmol/L, 10 μmol/L Ber or mmol/L Aspirin for 24 or 48 h. Then, whole-cell lysate was made in lysis buffer (1% Triton X-100, 50 mmol/L KCl, 25 mmol/L HEPES, pH 7.8, 10 μg/mL leupeptin, 20 μg/mL aprotinin, 125 μmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L sodium orthovanadate) by sonication. For NF-κB p65 detection, cytosolic and nuclear fractions were separated (first buffer: 10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 1.5 mmol/L $MgCl_2$, 0.1% NP4O, 1 mmol/L DTT; second buffer: 20 mmol/L HEPES pH 7.9, 420 mmol/L NaCl, 0.1 mmol/L EDTA, 1.5 mmol/L $MgCl_2$, 25% glycerol, 1 mmol/L DTT, 0.5 mmol/L PMSF. Protein concentrations were measured by BCA Protein Assay kit (Pierce, USA).The protein (50 μg) in 50 μL reducing sample buffer was boiled for 5 min, and resolved by SDS-PAGE for 2 h at 110 V. Then, the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane. After transfer, the PVDF membrane was washed with 25 mL Tris-buffered saline (TBS) for 5 min at room temperature. The membrane was incubated in 25 mL blocking buffer (1 × TBS, 0.1% Tween-20 with 5% non-fat dry milk) for 2 h at room temperature, and then washed three times for 5 min each with 15 mL TBS/0.1% Tween-20 (TBS/T). The membrane was incubated with primary antibody including IKKβ antibodies, phosphorylated IKKβ Ser^{181} antibodies, phosphorylated IRS-1 Ser^{307} antibodies (Cell Signaling, USA), IRS-1 antibodies, PI-3K p85 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GLUT4 antibodies (R&D Systems, USA) NF-κB p65 antibodies (Neomarkers, USA), β-actin antibodies (Lab Vision, USA) (at the appropriate dilution) in 10 mL primary antibody dilution buffer with gentle agitation overnight at 4℃, and then washed three times for 5 min each with 15 mL 10 × TBS/0.1% TBS/T. The membrane was incubated with HRP-conjugated secondary antibody (1:3000; Pierce) in 10 mL blocking buffer with gentle agitation for 2 h at room temperature, and washed three times for 5 min each

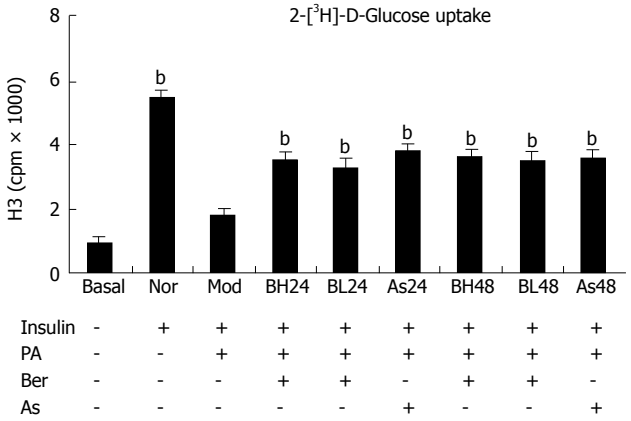


Figure 1 Insulin-induced 2-deoxy-[^{3}H]-D-Glucose uptake in 3T3-L1 adipocytes . 3T3-L1 preadipocytes (5×10^5/well) were differentiated to adipocytes in a 24-well plate. After serum-starvation in 0.2% BSA DMEM overnight, the cells were incubated in 0.2% BSA DMEM containing 0.5 mmol/L PA or (and) 1 μmol/L, 10 μmol/L Ber or 5 mmol/L Aspirin for 24 h or 48 h. Then, the cells were incubated in 1 mL KRP-HEPES without or with 100 nmol/L insulin for 30 min at 37℃ after washed three times in KRP-HEPES buffer. Next, the cells were incubated in 1 mL KRP-HEPES containing 0.5 μCi/mL 2-deoxy-D-[^{3}H] glucose for 10 min at 37℃. Finally, the cells were washed three times in ice-cold PBS and solubilized in 1ml of 0.1 mol/L NaOH for 2 h. Radioactivity was determined by liquid scintillation spectrometry. Nonspecific deoxyglucose uptake was measured in the presence of 20 μmol/L cytochalasin B and specific glucose uptake was detected from the subtracted total uptake. Three replicate wells were set up and each experiment was repeated three times. $^bP < 0.01$, *vs* Mod group.

with 15 mL TBS/T. Immunoreactive bands were detected with the ECL kit (Pierce) and immunoreactive bands on autoradiography films were scanned (Bio-Rad). Intensity of the immunoblot signal was analyzed quantitatively using Quantity One software (Bio-Rad).

Detection of NF-κB p65 proteins by laser scanning confocal microscopy (LSCM)

3T3-L1 preadipocytes were differentiated into adipocytes in a six-well plate. After serum-starvation in 0.2% BSA DMEM overnight, the cells were incubated in 0.2% BSA DMEM containing 0.5 mmol/L PA or (and) 10 μmol/L Ber or 5 mmol/L aspirin for 24 or 48 h. Cells were harvested and coverslips were fixed with fixed liquid (dehydrated alcohol: acetone; 1:1 v/v) for 10 min and 1% Triton X-100 for 5 min. After completely rinsing with water and washing with PBS, rabbit-anti-human NF-κB p65 antibody (1:100, 50 μL) was added drop wise and stored at 4℃ overnight. The following day, after washing with PBS, Cy-3-labeled sheep-anti-rabbit IgG was added drop wise, and the samples were incubated in a wet box in the dark at 37℃ for 30 min. After washing with PBS in the dark, diamidino phenylindole (DAPI) solution was added drop wise to counterstain the nucleus for 30 s (in the dark at room temperature, about 37℃). After washing with PBS and distilled water, 50% buffered glycerol was used for coverslip sealing. Image acquisition was achieved using an Olympus FV500 fluorescent microscope. An immersion objective lens was used to observe the samples. The excitation wavelengths of CY-3 and DAPI were 552 and 358 nm, respectively. The emission wavelengths were 565 and 460 nm, respectively. Ten randomly selected areas were taken from each coverslip to account for variations in the expression levels of NF-κB p65. Images were analyzed quantitatively by the HMIAS-2000 Imaging System, China.

Table 1 CCK-8 values in 3T3-L1 adipocytes

Group	*n*	CCK-8 value (Absorption495)
Nor	9	1.869 ± 0.050
Mod	9	1.848 ± 0.057[a]
BH24	9	1.830 ± 0.066[a]
BL24	9	1.846 ± 0.019[a]
As24	9	1.819 ± 0.141[a]
BH48	9	1.836 ± 0.047[a]
BL48	9	1.852 ± 0.050[a]
As48	9	1.803 ± 0.088[a]

[a]$P > 0.05$ *vs* Nor group.

Statistical analysis

Data are expressed as means ± SD. Statistical and graphical analysis was performed with SPSS version 10.0 software. Analyses were performed using an independent-samples *t* test where appropriate.

RESULTS

Berberine increases insulin-induced glucose uptake in 3T3-L1 adipocytes with insulin resistance

We used 3T3-L1 adipocytes as a cellular model to analyze the insulin signaling pathway. 3T3-L1 adipocytes were treated with PA (0.5 mmol/L, 24 h) to induce insulin resistance. Insulin-induced glucose uptake was measured to determine insulin sensitivity. The results showed insulin-induced glucose uptake was 5.6 times higher than that in the normal group, but was inhibited by as much as 67% after incubation with PA for 24 h. This is consistent with reports FFA induces insulin resistance in cell culture[10,11]. However, intervention with berberine or aspirin reversed the condition completely. Insulin-induced glucose uptake was increased by 82% and 93% after intervention with 1 and 10 μmol/L berberine for 24 h, and increased by 93% and 101% after intervention with berberine for 48 h, respectively. The data was performed in a dose- and time-dependent manner. In addition, the insulin-induced glucose uptake was increased by 109% and 97%, respectively, in the aspirin groups (Figure 1). The viability and number of cells might have decreased because of the toxic effects of 48-h aspirin treatment (Table 1).

Berberine increases the expression of IRS-1 and PI-3K p85 protein in 3T3-L1 adipocytes with insulin resistance

To understand the role of insulin signal transduction proteins in the mechanism by which berberine acts on FFA-induced insulin resistance, IRS-1, PI-3K p85 and GLUT4 protein abundance were detected with IRS-1, PI-3K p85 and GLUT4 antibodies by immunoblotting in 3T3-L1 adipocytes. The results showed that expression of IRS-1 and PI-3K p85 protein was reduced after incubation with FFA (0.5 mmol/L PA, 24 h) ($P < 0.01$) and this reduction was inhibited by berberine (1 or 10 μmol/L) or aspirin (5 mmol/L). The data was still performed in a

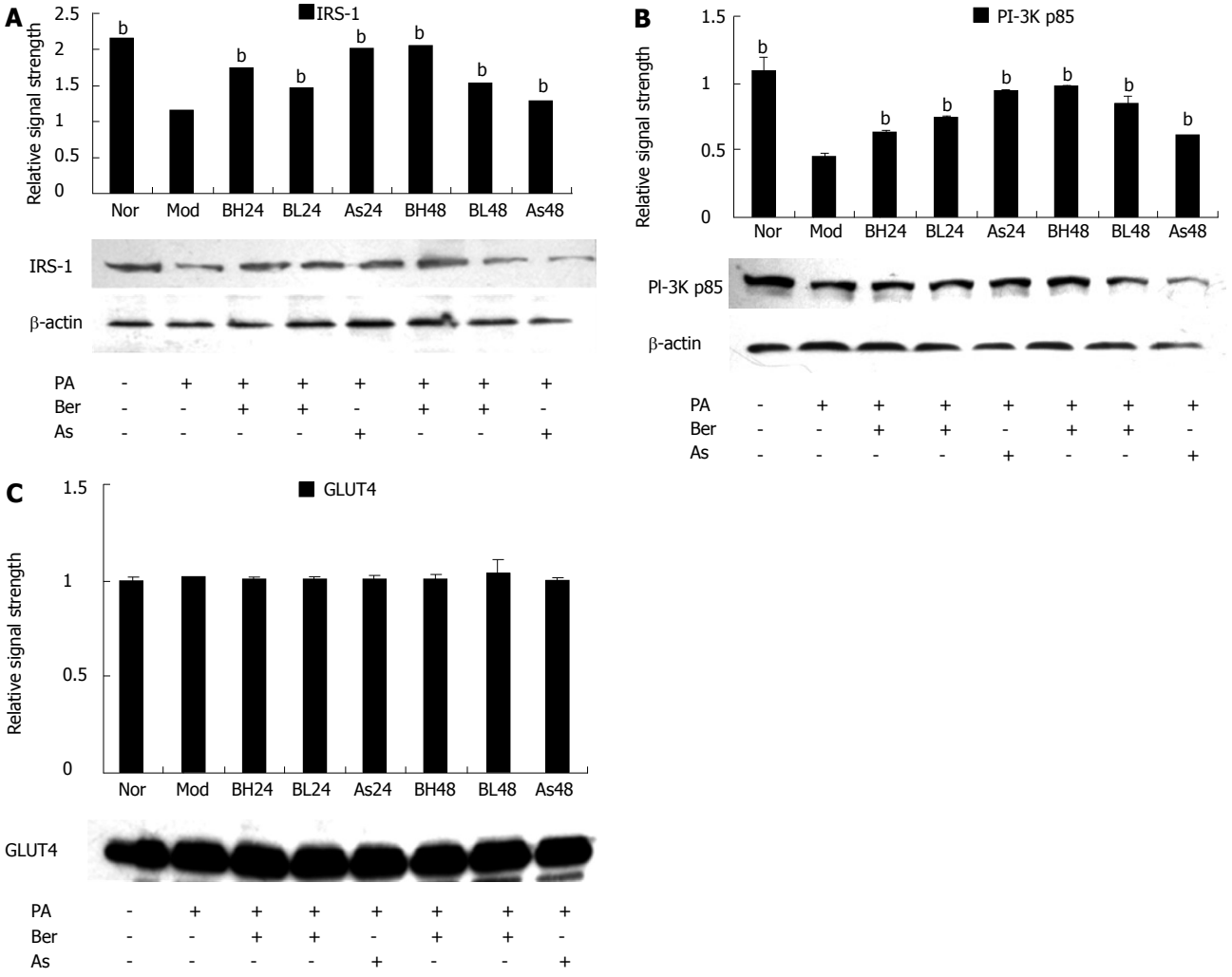


Figure 2 **A**: Effect of Ber on the IRS-1 Protein Expression in 3T3-L1 adipocytes. Fully differentiated cells were cultured for 12 h in serum-free DMEM with 0.2% BSA and then they were, respectively, cultured for 24 h in DMEM containing 0.5 mmol/L PA and 1% BSA (Mod); or cultured for 24 and 48 h in DMEM containing 0.5 mmol/L PA, 10 μmol/L Ber, 1% BSA (BH24, BH48); or in DMEM containing 0.5 mmol/L PA, 1 μmol/L Ber, 1% BSA for 24 h and 48 h (BL24, BL48); or cultured in DMEM containing 0.5 mmol/L PA, 5 mmol/L aspirin, 1% BSA for 24 h and 48 h (As24, As48); or in DMEM containing 1% BSA for 24 h (Nor). IRS-1 proteins were determined in the whole cell lysate by immunoblotting. Each experiment was repeated three times. $^{b}P < 0.01$ *vs* Mod group; **B**: Effect of Ber on the PI-3K p85 Protein Expression in 3T3-L1 adipocytes. Fully differentiated cells were cultured for 12 h in serum-free DMEM with 0. 2% BSA and then they were, respectively, cultured for 24 h in DMEM containing 0.5 mmol/L PA and 1% BSA (Mod); or cultured for 24 and 48 h in DMEM containing 0.5 mmol/L PA, 10 μmol/ L Ber, 1% BSA (BH24, BH48); or in DMEM containing 0.5 mmol/L PA,1 μmol/ L Ber, 1% BSA for 24 h and 48 h (BL24, BL48); or cultured in DMEM containing 0.5 mmol/L PA, 5 mmol/L aspirin, 1% BSA for 24 h and 48 h (As24, As48); or in DMEM containing 1% BSA for 24 h (Nor). Insulin was used at 100 nmol/L for 30 min. PI-3K p85 proteins were determined in the whole cell lysate by immunoblotting. Each experiment was repeated three times. $^{b}P < 0.01$ *vs* Mod group; **C**: Effect of Ber on the GLUT4 Protein Expression in 3T3-L1 Adipocytes. Fully differentiated cells were cultured for 12 h in serum-free DMEM with 0.2% BSA and then they were, respectively, cultured for 24 h in DMEM containing 0.5 mmol/L PA and 1% BSA (Mod); or cultured for 24 and 48 h in DMEM containing 0.5 mmol/L PA, 10 μmol/L Ber, 1% BSA (BH24, BH48); or in DMEM containing 0.5 mmol/L PA, 1 μmol/L Ber, 1% BSA for 24 h and 48 h (BL24, BL48); or cultured in DMEM containing 0.5 mmol/L PA, 5 mmol/L aspirin, 1% BSA for 24 h and 48 h (As24, As48); or in DMEM containing 1% BSA for 24 h (Nor). GLUT4 proteins were determined in the whole cell lysate by immunoblotting. Each experiment was repeated three times.

dose- and time-dependent manner ($P < 0.01$) (Figure 2A and B). However, there was no change in the expression of GLUT4 protein after the intervention of FFA, berberine and aspirin (Figure 2C).

Berberine inhibits FFA-induced IRS-1 Ser^{307} phosphorylation in 3T3-L1 adipocytes

Serine phosphorylation precedes IRS-1 degradation[12-14] and Ser^{307} phosphorylation of IRS-1 has become a molecular indicator of insulin resistance[15-18], therefore, we investigated the effect of berberine on IRS-1 Ser^{307} phosphorylation with phosphorylation-specific IRS-1 Ser^{307} antibody by immunoblotting. In 3T3-L1 adipocytes, PA (0.5 mmol/L, 24 h) may have induced IRS-1 Ser^{307} phosphorylation and berberine (1 or 10 μmol/L) or aspirin (5 mmol/L) were found to reduce IRS-1 Ser^{307} phosphorylation, and this reduction by berberine was dose- and time-dependent. These results suggest that Ser^{307} phosphorylation contributes to IRS-1 degradation and berberine inhibits FFA-induced IRS-1 Ser^{307} phosphorylation in 3T3-L1 adipocytes (Figure 3).

Berberine inhibits FFA-induced IKKβ Ser^{181} phosphorylation in 3T3-L1 adipocytes

It was reported that Ser^{307} of IRS-1 can be phosphorylated by either IKK or JNK[15,19], and IKK is more sensitive

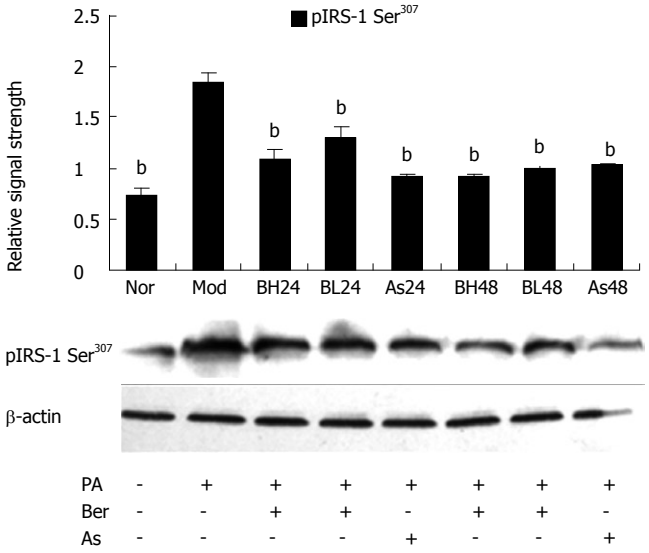


Figure 3 Effect of Ber on the IRS-1 Ser^{307} Phosphorylation in 3T3-L1 Adipocytes. Fully differentiated cells were cultured for 12 h in serum-free DMEM with 0.2% BSA and then they were, respectively, cultured for 24 h in DMEM containing 0.5 mmol/L PA and 1% BSA (Mod); or cultured for 24 and 48 h in DMEM containing 0.5 mmol/L PA, 10 μmol/ L Ber, (1% BSA, BH24) BH48; or in DMEM containing 0.5 mmol/L PA, 1 μmol/ L Ber, 1% BSA for 24 h and 48 h (BL24, BL48); or cultured in DMEM containing 0.5 mmol/L PA, 5 mmol/L aspirin, 1% BSA for 24 h and 48 h (As24, As48); or in DMEM containing 1% BSA for 24 h (Nor). IRS-1 phosphorylation was determined in the whole cell lysate by immunoblotting with the phosopho-spcific IRS-1(Ser^{307}) antibody. Each experiment was repeated three times. $^{b}P < 0.01$, *vs* Mod group.

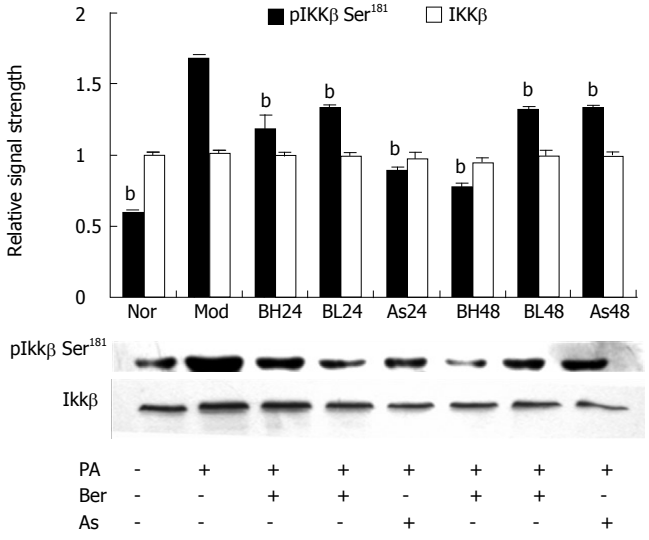


Figure 4 Effect of Ber on the IKKβ Ser^{181} Phosphorylation and IKKβ protein expression in 3T3-L1 Adipocytes. Fully differentiated cells were cultured for 12 h in serum-free DMEM with 0.2% BSA and then they were, respectively, cultured for 24 h in DMEM containing 0.5 mmol/L PA and 1% BSA (Mod); or cultured for 24 and 48 h in DMEM containing 0.5 mmol/L PA, 10 μmol/L Ber, 1% BSA, BH24, BH48; or in DMEM containing 0.5 mmol/L PA, 1 μmol/L Ber, 1% BSA for 24 h and 48 h (BL24, BL48); or cultured in DMEM containing 0.5 mmol/L PA, 5 mmol/L aspirin, 1% BSA for 24 h and 48 h (As24, As48); or in DMEM containing 1% BSA for 24 h (Nor). IKKβ phosphorylation and IKKβ protein expression were determined in the whole cell lysate by immunoblotting with the phosopho-spcific IKKβ (Ser^{181}) antibody and IKKβ antibody respectively. Each experiment was repeated three times. $^{b}P < 0.01$, as compared with Mod group.

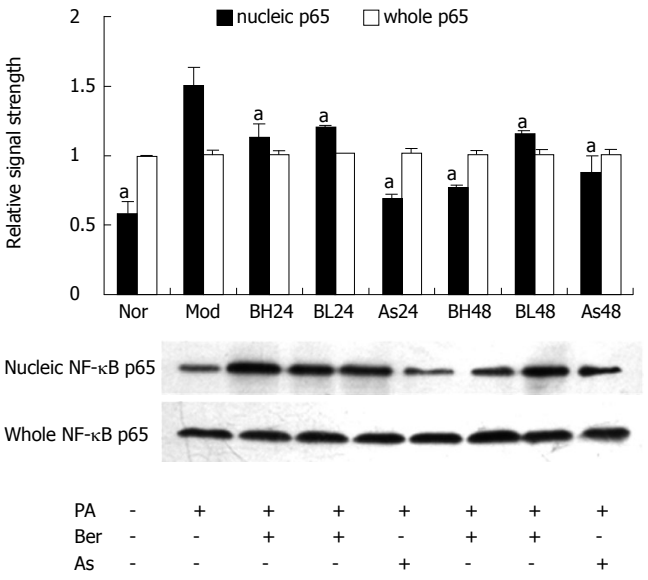


Figure 5 Effect of Ber on the whole NF-κB p65 and nucleic NF-κB p65 protein expression in 3T3-L1 Adipocytes. Fully differentiated cells were cultured for 12 h in serum-free DMEM with 0.2% BSA and then they were, respectively, cultured for 24 h in DMEM containing 0.5 mmol/L PA and 1% BSA (Mod); or cultured for 24 and 48 h in DMEM containing 0.5 mmol/L PA, 10 μmol/L Ber,1% BSA (BH24, BH48); or in DMEM containing 0.5 mmol/L PA, 1 μmol/L Ber, 1% BSA for 24 h and 48 h (BL24, BL48); or cultured in DMEM containing 0.5 mmol/L PA, 5 mmol/L aspirin, 1% BSA for 24 h and 48 h (As24, As48); or in DMEM containing 1% BSA for 24 h (Nor). whole NF-κB p65 and nucleic NF-κB p65 protein expression were determined in the whole cell lysate and nucleic cell lysate by immunoblotting respectively. Each experiment was repeated three times. $^{a}P < 0.05$ *vs* Mod group.

to FFAs. In mammalian cells, phosphorylation of Ser^{181} at the activation loop is essential for activation of the catalytic activity of IKK[20,21]. In this study, we investigated an effect of berberine on IKKβ serine^{181} phosphorylation and protein abundance of IKKβ with the phosphorylation-specific IKKβ Ser^{181} antibody and IKKβ antibody by immunoblotting. In 3T3-L1 adipocytes, PA (0.5 mmol/L, 24 h) may induce IKKβ Ser^{181} phosphorylation; berberine (1 μmol/L, 10 μmol/L) or aspirin (5 mmol/L) were found to reduce IKKβ Ser^{181} phosphorylation, with the the reduction by berberine in a dose- and time-dependent manner ($P < 0.01$). However, IKKβ protein abundance was unchanged during this study. These results suggest Ser^{307} phosphorylation of IRS-1 is associated with activation of IKKβ, and that berberine inhibits FFA-induced IKKβ Ser^{181} phosphorylation in 3T3-L1 adipocytes (Figure 4).

Berberine inhibits expression of FFA-induced nuclear NF-κB p65 protein in 3T3-L1 adipocytes

The NF-κB/Rel transcription factors are present in the cytosol in an inactive state, combined with the inhibitory IkB proteins. The key step in NF-κB nuclear translocation is mediated by IKKβ. In this study, we examined the effect of berberine on NF-κB p65 protein abundance obtained from whole-cell extracts and nuclear extracts with NF-κB p65 antibody, by immunoblotting. The results showed that, in 3T3-L1 adipocytes, PA (0.5 mmol/L, 24 h) may have induced nuclear NF-κB p65 protein, and berberine (1 or 10 μmol/L) or aspirin (5 mmol/L) were found to reduce expression of NF-κB p65 protein, and this reduction by berberine was dose- and time-dependent ($P < 0.05$). However, whole NF-κB p65 protein abundance was unchanged during this study (Figure 5).

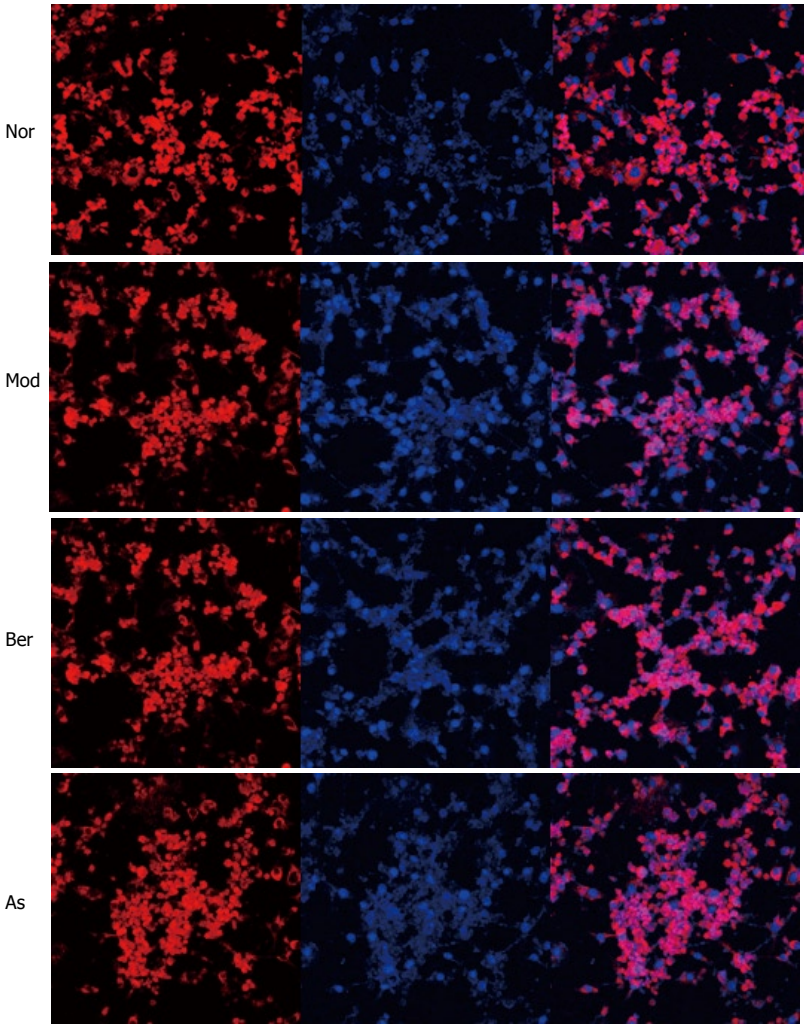


Figure 6 Fully differentiated cells were cultured for 12 h in serum-free DMEM with 0.2% BSA and then they were, respectively, cultured for 24 h in DMEM containing 1% BSA for 24 h (Nor); or cultured for 24 h in DMEM containing 0.5 mmol/L PA and 1% BSA (Mod); or cultured for 48 h in DMEM containing 10 μmol/L Ber, 0.5 mmol/L PA, 1% BSA (Berberine); or cultured for 24h in DMEM containing 5 mmol/L aspirin, 0.5 mmol/L PA, 1% BSA (Aspirin). Without FFA stimulation NF-κB p65 is predominately found in the cytoplasm, imparting red color (Nor). While FFA stimulation, NF-κB p65 is translocated into the nucleus, showing pink color (Mod). The addition of 10 μmol/L Ber or 5 mmol/L aspirin clearly inhibits the FFAs induced NF-κB p65 translocation, as there is hardly any nuclear p65 staining found and in the figure, both red and pink colors can be observed (Berberine and Aspirin).

Berberine inhibits nuclear translocation of NF-κB p65 protein in 3T3-L1 adipocytes

To test the hypothesis that berberine inhibits expression of FFA-induced NF-κB p65 protein in 3T3-L1 adipocytes, immunofluorescence by LSCM was used to investigate the distribution of NF-κB p65. Nuclei were stained with DAPI (blue) and NF-κB p65 was stained with CY-3 (red). The confocal superimposition of the two fluorescence stains yielded a pink color, which indicated that NF-κB p65 was translocated into the nuclei. The results showed that, without FFA stimulation, NF-κB p65 was predominately found in the cytoplasm, and imparted a red color (normal group), while with FFA stimulation, NF-κB p65 was translocated to the nucleus, and showed a pink color (model group). The addition of 10 μmol/L berberine or 5 mmol/L aspirin clearly inhibited the FFA-induced NF-κB p65 translocation; there was hardly any nuclear p65 staining found, and in the figure, both red and pink colors can be observed (indicating berberine and aspirin), which is consistent with the Western blot analysis results (Figure 6).

DISCUSSION

Berberine is an isoquinoline alkaloid extracted from *Rhizoma Coptidis*, and it was initially used as a heat-clearing and detoxicating agent and as an anti-inflammatory drug in clinical practice. Further pharmacological studies have found that the effects of berberine include improving insulin resistance, lowering blood sugar, and correcting disorders of lipid metabolism[1-4]. Based on the effects of berberine on diabetes and the new notion that type 2 diabetes is essentially a disease of chronic inflammation, we make hypothesis if the anti-inflammation effect of Ber is related to the effect of insulin-resistance improvement.

Recently, it has been reported that salicylic acid, a traditional anti-inflammatory agent, reverses obesity- and diet-induced insulin resistance by inhibiting IKKβ[5,6]. Many studies have shown insulin signal transduction proteins are regulated, not only by tyrosine phosphorylation, but also by Ser/Thr phosphorylation. IKKβ, a Ser/Thr protein kinase, is closely related to the development of insulin resistance and is known to be a new target for the improvement of insulin resistance[5]. Is IKKβ a potential molecular target for berberine for improving insulin resistance and relieving inflammation?

In our previous studies, we have established a murine model of insulin resistance by injection of small doses of streptozotocin via the tail vein plus oral administration of high-fat and high-caloric diet. After treatment with berberine for 10 wk, the levels of fasting blood glucose and insulin were substantially lowered; the oral glucose tolerance test was conspicuously improved; serum inflammatory factors IL-1β, IL-6, TNF-α, acute-phase

reactive protein (CRP), and adhesion molecules (ICAM-1, VCAM-1) were obviously lowered; and at the same time, tyrosine phosphorylation of insulin receptor β subunit, tyrosine phosphorylation of IRS-1, expression of IRS-1, GLUT4, PI-3K protein, and translocation of GLUT4 protein were significantly elevated in adipose and muscular tissues, compared with those in the model group. This suggests berberine reduces inflammation, lowers blood glucose and improves insulin resistance[22-24].

In the present study, we investigated the underlying mechanisms of the effects of berberine on insulin resistance and inflammation *in vitro*. Insulin resistance in 3T3-L1 adipocytes was induced by incubation with PA (0.5 mmol/L, 24 h), as judged by a 67% decrease in insulin-stimulated glucose uptake. This was reversed by prior incubation of cells with berberine or aspirin. The expression of IRS-1 and PI-3K p85 proteins was reduced after PA incubation and this reduction was inhibited by intervention with berberine (1 or 10 μmol/L) or aspirin (5 mmol/L). However, there was no change in GLUT4 abundance after incubation with FFAs, berberine and aspirin. The results suggest inhibition of insulin-induced glucose uptake is caused by reduction of IRS-1 and PI-3K, not GLUT4. Thus, IRS-1 and PI-3K p85 protein, but not GLUT4, might be involved in the effects of berberine on insulin resistance in 3T3-L1 adipocytes.

It has been shown Ser^{307} phosphorylation contributes to IRS-1 degradation in hepatocytes in response to insulin[25]. In our study, PA may have increased IRS-1 Ser^{307} phosphorylation, while berberine or aspirin were found to reduce IRS-1 Ser^{307} phosphorylation. It can be inferred that the same mechanism contributes to FFA-induced degradation of IRS-1 in adipocytes, and Ser^{307} phosphorylation of IRS-1 was inhibited by berberine. Since Ser^{307} of IRS-1 can be phosphorylated by IKK[15,19] and phosphorylation of Ser^{181} at the activation loop is essential for activation of the catalytic activity of IKK[20,21], we observed the expression of IKKβ Ser^{181} phosphorylation in 3T3-L1 adipocytes. PA may have increased IKKβ Ser^{181} phosphorylation, and this was reversed by prior incubation of cells with berberine or aspirin. It was demonstrated that IKKβ Ser^{181} phosphorylation may contribute to IRS-1 Ser^{307} phosphorylation in response to FFAs, and berberine inhibited FFA-induced IKKβ Ser^{181} phosphorylation in 3T3-L1 adipocytes.

It is known the NF-κB/Rel transcription factors are present in the cytosol in an inactive state, complexed with the inhibitory IkB proteins, and that NF-κB nuclear translocation is mediated by IKKβ[26,27]. In our study, we detected NF-κB p65 protein in whole-cell and nuclear extracts, and investigated the distribution of NF-κB p65. It has been shown that in 3T3-L1 adipocytes, PA may increase the expression of NF-κB p65 protein, while berberine or aspirin reduce the expression of NF-κB p65 protein. However, NF-κB p65 protein abundance was not changed during this study. LSCM demonstrated significant translocation of NF-κB p65 into the nucleus after stimulation with PA. The translocation was inhibited by berberine or aspirin, which is consistent with the results of Western blot analysis. Taken together, the data suggest that berberine inhibits translocation of NF-κB p65 into the nucleus in 3T3-L1 adipocytes.

In summary, our study demonstrated potential links between chronic inflammation and insulin resistance. IKKβ may be an important target for this link. Increased IKKβ activity promotes insulin resistance, while reduction in IKKβ activity significantly improves insulin sensitivity. In the signaling pathway, FFAs activate IKKβ, which leads to Ser^{307} phosphorylation in IRS-1 and translocation of NF-κB p65 into the nucleus[28,29]. On the one hand, serine phosphorylation is responsible for a reduction in tyrosine phosphorylation of IRS-1, which leads to a reduction in IRS-1 protein and insulin resistance. On the other hand, translocation of NF-κB p65 into the nucleus may enhance the production of inflammatory molecules such as TNFα. The positive feedback loop may perpetuate a cycle of low-level inflammatory signaling that leads to insulin resistance. Our findings were consistent with those reported by Gao *et al*[30]. Thus, inhibitors of IKKβ, such as aspirin and berberine, through the same signaling pathway, have significant effects on insulin resistance. Therefore, we conclude berberine might achieve its anti-inflammatory and insulin-resistance-improving effects by inhibiting phosphorylation of IKKβ Ser^{181}.

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COMMENTS

Background

Berberine was initially used as a heat-clearing and detoxifying agent and an anti-inflammatory drug in clinical practice. Further pharmacological studies have found the effects of berberine include improving insulin resistance, lowering blood sugar and correcting disorders of lipid metabolism *in vitro* and *in vivo*. It has been shown that type 2 diabetes is essentially a disease of chronic inflammation. The question remains if the anti-inflammatory effect of berberin is related to the effect of insulin resistance improvement.

Research frontiers

Recently, it has been reported that salicylic acid, a traditional anti-inflammatory agent, may reverse obesity- and diet-induced insulin resistance by inhibiting IKKβ. Many studies have shown insulin signal transduction proteins are regulated, not only by tyrosine phosphorylation, but also by Ser/Thr phosphorylation. IKKβ, a Ser/Thr protein kinase, is closely related to the development of insulin resistance and is known as a new target for the improvement of insulin resistance. Is IKKβ a potential molecular target for berberine for improving insulin resistance and relieving inflammation?

Innovations and breakthroughs

Our research has established a model of insulin resistance induced by FFA in 3T3-L1 adipocytes, and we have observed the effects of berberine on the expression of inflammatory molecules such as IKKβ, NF-κB and insulin-signal-transduction-related molecules such as IRS-1, PI-3K and GLUT4. These results showed IKKβ may be an important target for potential links between chronic inflammation and insulin resistance. Berberine might achieve its anti-inflammatory and insulin-resistance-improving effects by inhibiting phosphorylation of IKKβ Ser^{181}.

Applications

Our study showed IKKβ may be a potential molecular target for berberine when improving insulin resistance and relieving inflammation. The use of berberine as

an inhibitor of IKKβ provides us with a new approach for the treatment of non-infectious chronic diseases, such as type 2 diabetes, obesity, hypertension, atherosclerosis and ischemic cerebrovascular disease.

Terminology

IKKβ: IKK is a multi-subunit protein kinase. There is strong biochemical and genetic evidence that the IKK complex consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ. However, of the two catalytic subunits, only IKKβ is essential for NF-κB activation in response to pro-inflammatory stimuli. Many studies have shown that IKKβ, a Ser/Thr protein kinase, is closely related to the development of insulin resistance, and is known as a new target for the improvement of insulin resistance.

Peer review

This study investigated the influence of berberine on FFA-treated 3T3-L1 cells. Similar data have not been published so far and the figures are clearly presented.

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CLINICAL RESEARCH

H pylori (*Cag*A) and Epstein-Barr virus infection in gastric carcinomas: Correlation with *p53* mutation and c-Myc, Bcl-2 and Bax expression

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Abstract

AIM: To investigate the interrelationship between *H pylori* and Epstein-Barr virus (EBV) infection in the gastric carcinogenesis having in focus the *p53* mutation and the c-Myc, Bcl-2 and Bax expression.

METHODS: seventy-one gastric carcinoma tissues were assessed by polymerase chain reaction (PCR) for *H pylori* and *in situ* hybridization for EBV. c-Myc, Bcl-2 and Bax expression were detected by immunohistochemistry and single-stranded conformational polymorphism (SSCP) for *p53* mutation.

RESULTS: The positivity rates for *H pylori* and EBV were 94.4% and 8.45%, respectively. The majority of the cases displayed only the *H pylori* presence. All EBV positive cases were also *H pylori* positive. None infectious agent was observed in 5.55% of the cases. The intestinal type tumor was more frequent in the co-infected and non-infected groups. The female predominated in the non-infected group showing statistical significance (70.4% *vs* 29.6%, $P = 0.039$). The Bcl-2 was only detected in the group exclusively infected by *H pylori*. However, c-Myc and Bax were detected in the three groups but with a low frequency in the co-infected group. Mutation of *p53* was present in all groups, with the highest frequencies in the *H pylori* positive groups.

CONCLUSION: The frequency of *H pylori* infection in gastric carcinomas was high. The presented data indicated that gastric carcinogenesis has different pathways depending of the presence of the two investigated infectious agents, suggesting a possible involvement of *H pylori* with apoptotic process. The low expression of c-Myc and Bax in the EBV-positive groups suggests that EBV may inhibit the expression of these proteins. Nevertheless, *p53* mutation shows to be a relevant alteration, independent of both infectious agents.

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Key words: Gastric carcinoma; *Helicobacter pylori*; Epstein-Barr virus, *p53*, Bax, Bcl-2, c-Myc

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INTRODUCTION

Gastric cancer is the fourth most common cancer and the second cause of cancer-related death worldwide[1]. There is substantial international variation in the gastric cancer incidence with the highest rates reported from Korea, Japan and Eastern Asia. Other high incidence areas include Eastern Europe and parts of Latin America, while Western Europe and the United States of America generally have low incidence rates. The global burden of gastric cancer is shifting rapidly from the developed world to the developing world[2].

Over 95% of stomach tumors are adenocarcinomas which are subdivided into two main histological types: intestinal type and diffuse type. The intestinal type is

related to corpus-dominant gastritis with gastric atrophy and intestinal metaplasia, whereas the diffuse type usually originates in pangastritis without atrophy[1,2].

The two main anatomic sites of gastric adenocarcinoma are proximal (cardia) and distal (noncardia). The frequency of the theses tumor sites varies in populations from different geographic locations, racial and socio economic groups. They may differ in genetic susceptibility, pathologic profile, clinical presentation and prognosis. The observed differences between gastric cancers by anatomic sites suggest that they are distinct diseases with different etiologies. Although the distal cancer is usually more frequent, a decreased incidence has been observed in this site while the incidence of the proximal site tumors have increased since the 1970's especially among males in the Western countries[1,3,4].

There are several etiologic factors involved in gastric cancer such as low socioeconomic status, diet, hereditary factors and *H pylori* infection, as the most cited in studies about this issue. Besides the *H pylori*, Epstein-Barr virus (EBV) infection is also associated with development of gastric cancer[1,2,6].

The discovery of *H pylori* infection in the early eighties proved a turning point in the understanding of the gastric cancer pathogenesis. While the link between *H pylori* and peptic ulcer was established soon after the successful culture of the bacteria, the association with gastric cancer remained doubtful for almost a decade before credible evidence could be presented. The major reason for this delay was the inability to demonstrate the presence of active infection in gastric cancer tissue[1,2]. Despite the recognition of the association between *H pylori* and gastric cancer the pathogenic mechanism involved in this process is still not understood.

In spite of the wide bibliography regarding to the other tumors, the first report about the association of the EBV with gastric carcinomas was in a case of lymphoepithelial-like gastric carcinoma[7]. Soon after, it was identified in common gastric carcinomas[8]. EBV infection is found in approximately 10% of ordinary gastric adenocarcinoma cases; however most of the mechanisms used by the virus to control this process are still unknown. Recent studies have shown that the expression pattern of EBV-encoded genes in gastric carcinoma is different from that in Burkitt's lymphoma and nasopharyngeal carcinoma (NPC), suggesting that the oncogenic mechanism of the EBV in gastric carcinoma may be unique[6,9,10].

In attempt to understand the carcinogenic process triggered by these two infectious agents, some works have looked for alteration in genes/proteins that these agents can target, and therefore, pointing possible pathogenic pathways. In this sense, proteins involved with cell cycle or apoptosis process emerge as a candidate for having a crucial role in the development of the neoplasms. Some of these, like c-Myc, *p53* and the apoptotic family members such as Bcl-2 and Bax, deserve attention for playing a key function in the cell proliferation and cell fate, besides they already have shown involvement in the tumorigenesis process in a variety of tumors, including gastric cancer[11-13].

Although the proteins mentioned above have different levels of alteration in gastric carcinoma, the association with *H pylori* or EBV is controversial[14-16]. Additionally, there is just one paper related *H pylori* and EBV infection with concomitant analysis, in gastric carcinomas, in which, proteins involved in cell cycle and apoptosis process were also evaluated[10]. So, the aim of the present study was to investigate the interrelationship between *H pylori* and EBV infection in the gastric carcinogenesis having in focus the *p53* mutation, and the expression of c-Myc, Bcl-2 and Bax.

MATERIALS AND METHODS

Clinical specimens

The present study was approved by the ethic committee from Federal University of Ceará and all subjects signed informed consent prior to inclusion. Samples from eighty-two patients with gastric carcinoma were collected from two hospitals of the Ceará State, Brazil: Walter Cantídeo Hospital at Federal University of Ceará and Saint House of Mercy in Fortaleza, both located in Fortaleza, the capital of the State. A representative formalin-fixed tumor specimens embedded in paraffin blocks was selected and histological sections (5 μm) were subjected to immunohistochemistry and *in situ* hybridization (ISH). Clinico-epidemiological data were collected from the medical reports. Upon gastrectomy, fragments of tumor were collected and subjected to DNA extraction and Polymerase Chain Reaction (PCR) to detect *urease C* gene of *H pylori* and *p53* mutation.

DNA extraction

DNA was extracted from frozen tumor tissue just when the fragment presented more than 80% of tumor cells. The genomic DNA was extracted using cetyltrimethyl ammonium bromide (CTAB) adapted from the method of Gary[17]. Although we have collected samples from 82 patients, DNA extraction was done in 71 samples because the others did not reach the established percentage of tumor cells.

Detection of H pylori and the presence of cagA gene

The PCR was done to detected *H pylori* infection because this technique is sensitive and specific and it was used by many studies[6,18,38].

The *H pylori* infection was detected by amplification of *urease C* gene using primers for PCR, described by Lage *et al*[18]. For the *H pylori*-positive samples, the presence of the *cag*A gene was identified using the primers described by Domingo *et al*[19]. PCR mixtures for amplification of both genes were prepared in a volume of 25 μL containing 0.4 μmol/L of each primer; 1.5 μmol/L $MgCl_2$; 0.8% Tween 20 and 1 μL of DNA sample. PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining. Sample was considered *H pylori* positive when an *ure*C fragment of 294bp was amplified while *cag*A gene was positive when a fragment of 297bp was detected. As positive control we used DNA known *H pylori* positive and as negative control we used distillated water.

RNA in situ hybridization

The presence of the EBV was identified by the expression of EBV-encode small RNA-1 (EBER1), the most

abundant viral product in latently infected cells. RNA *in situ* hybridization was performed using a 30bp biotinylated probe complementary to the RNA EBER1 described by Shibata and Weiss[8]. Briefly, after the deparaffinization and rehydration, endogenous peroxidase was blocked with 3% H_2O_2 solution. Enzymatic digestion was performed with proteinase K (0.02 μg/μL final concentration) for 13 min. Prehybridization was done with pre-hybridization solution (Denhardt's solution [3.5X]; SSC [4.5X]; EDTA [0.0075 mol/L]; SDS [0.35%]; NaH2PO4 [0.75 mol/L]; dextran sulfate [10%]) for 60 min at 37℃. Soon after, the slides were incubated overnight at 37℃ with hybridization solution containing 0.3 ng/μL of the probe. After washing with 2X SSC buffer, the signal was amplified using anti-biotin antibody (clone BK, mouse, dilution 1:20) and biotinylated anti-immunoglobulin antibody (polyclonal, rabbit, dilution 1:100). Detection was accomplished using streptavidin-biotin-peroxidase method and the reaction was developed with 3, 3'-diaminobenzidine chromogen. All antibodies were purchased from DakoCytomation®. The slides were counterstained with Harris hematoxylin. A case of nasopharyngeal carcinoma was used as positive control in each reaction, as well as a negative control by omitting the probe. Nuclei with a brown or black staining were considered positive.

Immunohistochemistry

The expression of the proteins Bcl-2, Bax and c-Myc was detected according to the method described by Hsu *et al*[20]. After the deparaffinization and rehydration, antigen retrieval was carried out by microwave-treatment of the slides for 15 min in 10 mmol/L citrate buffer solution (pH 6.0). Endogenous peroxidase activity was blocked with 3% H_2O_2 solution. Primary antibodies were incubated for 16 h at 4℃-8℃ in a humid chamber. All antibodies were purchased from DakoCytomation®. The reaction was detected with LSAB + system (DakoCytomation®) according to the manufacturer's recommendation.

Histopathological and staining analysis

The histological classification (Laurén) was obtained from the medical reporters and was confirmed by a professional pathologist of the team. The slides were evaluated by three experienced technicians independently using light microscopy at a magnification of 400X. In the *in situ* hybridization and immunohistochemistry techniques, the results were expressed as percentage of positive cases. In the immunohistochemistry analysis, only the cases with $\geqslant$ 5% of stained tumor cells were considered as positive while in the *in situ* hybridization, any nuclear staining in tumor cells were considered. For all analyses, at least 1000 tumor cells were counted in high power fields.

Analysis of p53 mutation

To detection of mutation in the *p53* gene was used Single-Stranded Conformational Polymorphism (SSCP) test. Exons 5-9 of *p53* gene were amplified by PCR using four pairs of the primes described by Murakami *et al*[21]. Each PCR reaction for a total of 25 μL final volume consisted of 0.2 mmol/L of the four deoxynucleotide triphosphate, 1.5 mmol/L $MgCl_2$, 0.4 μmol/L of each primer and 0.5 U of Taq DNA polymerase (Invitrogen®). The cycling temperature included an initial temperature at 95℃ for 5 min, followed by 35 cycles of 95℃ for 1 min, 1 min for annealing temperature (55℃ for exons 5 and 8/9; 63℃ for exons 6 and 7), and extension at 72℃ for 1 min. For SSCP analysis, five or six microliters of each PCR products were mixed with a equal volume of stop solution (95% of formamide, 20 mmol/L EDTA, 0.05% of xylene cyanol and 0.05% of bromophenol blue), heated at 95℃ for 5 min and immediately placed on ice and loaded onto a gel containing 12.5% acrylamide (GenePhor™, Amersham Pharmacia Biotech). Electrophoresis was performed using Electrophoresis Unit GenePhor (GE-Healthcare), at 4℃ for 3 h. The patterns of the bands were then visualized by a DNA Silver Staining Kit (Amersham Pharmacia Biotech).

Statistical analysis

The analyses were carried out using the statistical programs EPINFO® 6.04 d version and SPSS® 12.0. Statistically significant differences were evaluated by Chi square test (χ^2). The results were considered as statistically significant when *P*-values were less than 0.05.

RESULTS

Among the analyzed cases, 57 were males and 25 were females. The mean age was 56.5 years (range, 23 to 90 years). Among 71 selected patients, males were a majority [70.4% (50/71)] and 49.3% (35/71) were more than 65 years old. Regard to the Lauren's classification, the intestinal type tumors demonstrated a slightly higher frequency (59.2%).

Detection of H pylori, EBV and markers

The *H pylori* infection was detected by PCR in 67 out of 71 (94.4%) gastric carcinomas. The correlation with sex showed a statistically significant difference $P = 0.041$ (Mantel-Haenszel), in this analysis, since almost all males were *H pylori* positive [98% (49/50)] while the females had a slightly lower prevalence [85.7% (18/21)]. Concerning the gastric regions, the rate of *H pylori* infection in the antrum was higher [55.22% (37/67)] than that of the cardia [28.35 (19/67)] and body [14.92% (10/67)]. The pathogenic genotype of *H pylori* (*cag*A +) was found in 62.7% (42/67) of the cases. Among these cases the intestinal type tumors was predominant [61.9% (26/42)].

The frequency of EBV infection was 8.45% (6/71). All EBV-positive cases were males and half of them were located in the gastric antrum [50% (3/6)]. Concerning the markers, the frequency of Bcl-2, Bax, c-Myc and of the *p53* mutation was 5.63% (4/71), 54.92% (39/71), 42.25% (30/71) and 73.23% (52/71), respectively. An EBV positive case is shown in Figure 1A, while the Figure 1B-D show the Bcl-2, Bax and c-Myc immunodetection, respectively. The Figure 2 is a SSCP gel showing the migration pattern of exons 5 and 7 of *p53* gene.

Relationship between H pylori, markers and p53 mutation

The *cagA* gene was detected in 62.7% (42/67) of the cases with *H pylori* infection. The relationship between

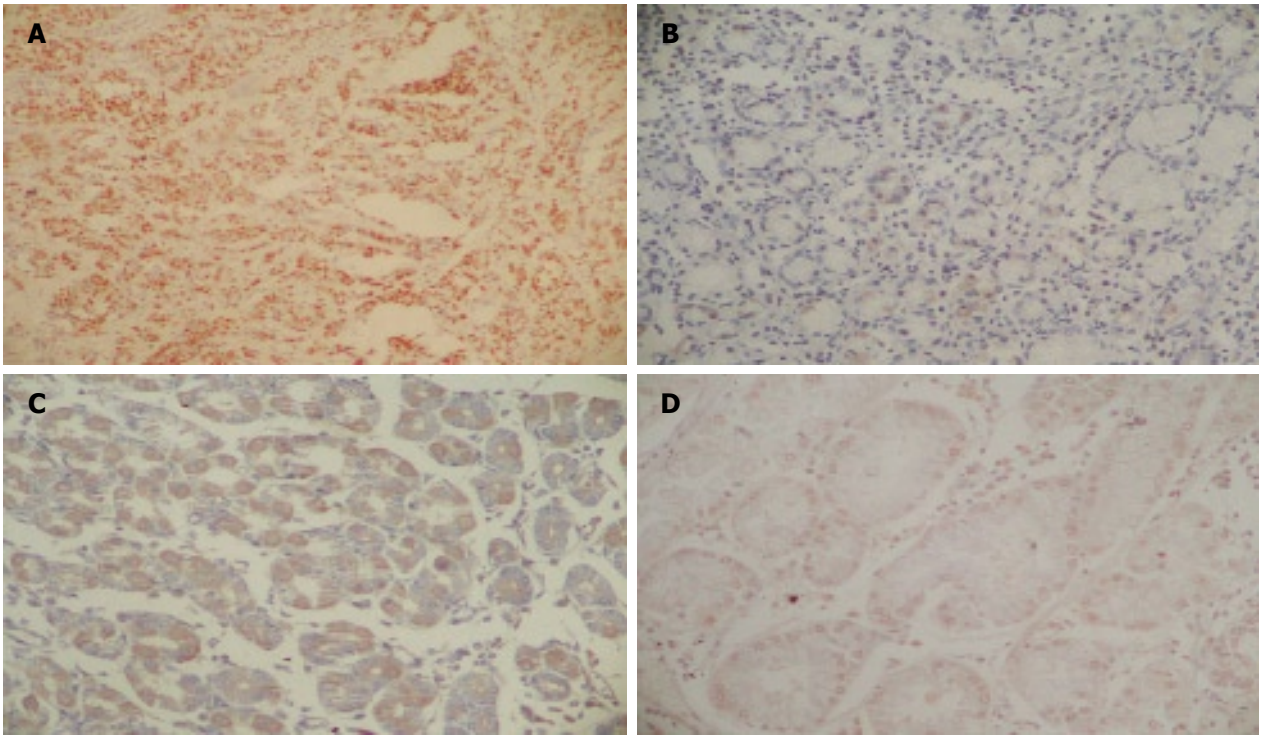


Figure 1 **A**: *In situ* hybridization detection of EBV RNA in gastric cancer (X400); **B**: Bcl-2 expression in gastric carcinoma (× 400); **C**: Bax expression in gastric carcinoma (×400); **D**: c-Myc expression in gastric carcinoma (× 400).

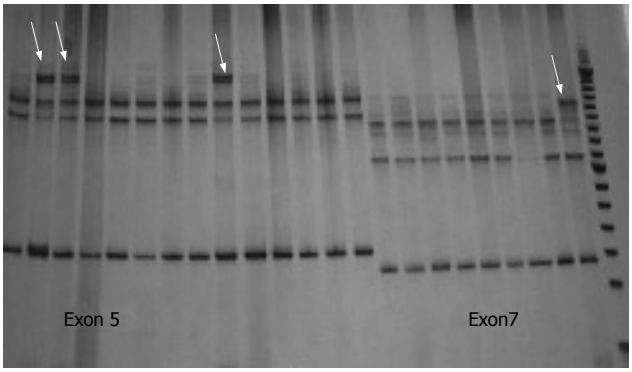


Figure 2 SSCP gel of *p53* gene. The arrows show the different migration patterns of exon 5 (lines 1 to 14) and 7 (lines 15 to 23).

Bcl-2, Bax and c-Myc was statistically not significant. The relationship of the *cag*A(+) with the *p53* mutation reached statistical significance ($P = 0.035$).

Relationship between EBV and H pylori

To investigate a possible relation between *H pylori* and EBV infection in the gastric tumorigenesis, the cases were divided in three groups: Hp(+)/EBV(+); Hp(+)/EBV(-) and Hp(-)/EBV(-). These groups accounted for 8.45% (6/71), 86% (61/71) and 5.55% (4/71) of the total, respectively. No EBV-positive case was found without *H pylori* infection.

The Table 1 summarizes the clinico-pathological data of the groups. The difference in gender distribution was statistically significant ($P = 0.039$), and, although overall the males predominated, in the Hp(-)/EBV(-) group the females were more frequent (75%). Furthermore, this group was represented by the most advanced ages. The intestinal type was more frequent in the Hp(+)/EBV(+) and Hp(-)/EBV(-) groups, although the distribution failed to reach statistical significance ($P = 0.339$), while in the group with only *H pylori* infection both histological types, intestinal and diffuse, occurred at the same rate.

In the two groups in which the *H pylori* were present, the *cag*A genotype predominated in tumors of the intestinal type. In the Hp(+)/EBV(+) group, 2 out of 3 tumors of Ⅳ stage were *cag*A(-), on the other hand, in the group with only *H pylori* infection, most of the tumors (6/9) in the same stage were *cag*A(+).

The diffuse type tumors, notably in the Hp(+)/EBV(+) and Hp(-)/EBV(-) groups, were in advanced stages (ⅢB and Ⅳ), while this was not the case in the intestinal type tumors. However, the correlation of the Lauren's classification with tumor stage was never significant. The diffuse type tumors of the Hp(+)/EBV(-) group had similar prevalence of *cag*A (+) and *cag*A (-) strains of *H pylori*. There was not any significant association between diffuse type and the tumor sites.

The Figure 3 shows the tumor stage distribution among the groups. Despite the small size of the groups with both or no infectious agents, it was possible to observe that the Ⅳ stage was the most represented stage in all groups. Even so, the earlier stage was present in the three groups. Not statistical significance was obtained in this analysis ($P = 0.179$).

The distribution of the studied markers according to the groups is presented in Figure 4**.** Although the expression of Bcl-2 was low, it was only observed in tumors with *H pylori* infection alone. Nevertheless, high expression of Bax and c-Myc was observed in the groups

Table 1 Correlation between clinico-pathological data, detection of EBV and *H pylori* and the pathogenic genotype (*cagA*) of *H pylori* *n* (%)

Clinico-pathological data	Total	Groups			*P*-value
		Hp(+)/EBV(+) (*n* = 6)	Hp(+)/EBV(-) (*n* = 61)	Hp(-)/EBV(-) (*n* = 4)	
Sex					*P* = 0.039
Males	50 (70.4)	6 (100)	43 (70.5)	1 (25.0)	
Females	21 (29.6)	0 (0)	18 (29.5)	3 (75.0)	
Age (yr)					*P* = 0.585
0-44	6 (8.5)	1 (16.6)	4 (6.56)	1 (25.0)	
45-54	12 (16.9)	1 (16.6)	11 (18.0)	0 (0.0)	
55-64	18 (25.4)	1 (16.6)	17 (27.8)	0 (0.0)	
> 65	35 (49.3)	3 (50)	29 (47.5)	3 (75.0)	
Histological types					*P* = 0.339
Intestinal	42 (59.2)	5 (83.4)	34 (55.7)	3 (75.0)	
Diffuse	29 (40.8)	1 (16.6)	27 (44.2)	1 (25.0)	
Anatomic site					*P* = 0.799
Cardia	19 (26.8)	2 (33.4)	17 (27.8)	0 (0.0)	
Body	11 (15.5)	1 (16.6)	9 (14.8)	1 (25.0)	
Antrum	40 (56.3)	3 (50)	34 (55.7)	3 (75.0)	
Multiple sites	1 (1.4)		1 (1.7)		
EBV					
ISH (+)	6 (8.4)	-	-	-	
ISH (-)	65 (91.5)	-	-	-	
H pylori					
PCR (+)	67 (94.4)	-	-	-	
PCR (-)	4 (5.6)	-	-	-	
H pylori PCR (+)					*P* = 0.833
*cag*A (+)	42 (62.7)	4 (66.6)	38 (62.3)	-	
*cag*A (-)	25 (37.3)	2 (33.3)	23 (37.7)	-	

Hp(+)/EBV(-) and Hp(-)/EBV(-), when compared with the coinfected tumors. A high frequency of the *p53* mutation was observed in all groups, especially Hp(+)/EBV(+) and Hp(+)/EBV(-). None of the markers significantly correlated with any of the groups.

DISCUSSION

H pylori is known to be a carcinogen agent of gastric cancer. The major advance in this field came with the recognition that chronic *H pylori* infection induces physiological and morphological changes within the gastric environment increasing the risk for neoplastic transformation. Now, it is widely accepted that chronic *H pylori* infection induces hypochlorhydria and gastric atrophy, which are precursors of gastric cancer[1,2]. Recently, it was found that EBV is also linked to the development of a portion of gastric carcinomas. Since then, several studies have been carried out about EBV carcinogenic role in these tumors. The relationship between EBV infection and gastric cancer has been demonstrated by strong pieces of evidence such as the monoclonality of the viral genome and its presence in almost all tumor cells, indicating that the infection had occurred before malignant transformation and that the tumor cells had been originated from an infected primary cell[6,8,9].

In this study, we assessed the status of EBV by ISH and *H pylori* infection by PCR in 71 cases of gastric carcinomas. The frequency of *H pylori* infection (94.4%) was higher than previous studies, where it ranged from 34.1% to 92%. These differences can be due to: (a) the sensitivity of the screening technique, since most previous studies used serology, rapid urease test and/or histological evaluation; (b) and the fact that these studies were performed in different world regions and it is known that the *H pylori* infection is more frequent in developing countries[8,16,22-24]. On the other hand, EBV was detected in 8.45% of our cases. Although low, this prevalence is in agreement with reported results from various world regions, which vary from 2% to 23.6%[6,9,14,24,25]. The epidemiological data are also in accordance with previous studies. The male-female ratio was 2:1 and the majority of the patients were older than fifty five[1,2]. Like in other studies, the intestinal type was more frequent than the diffuse type[1,8].

Despite the known importance of *H pylori* and EBV in the gastric cancer etiology, few studies have focused on the interrelationship of these two agents in gastric cancer cases. Thus, we investigated the presence of both *H pylori* and EBV, in parallel with the histopathological features, the status of the tumor suppressor p53, the expression of the c-Myc oncogene and the apoptotic proteins Bcl-2 and Bax, which have a key role in the tumor development. To do so, patients were divided in three groups based on the presence or absence of the infectious agents.

In our analysis, we observed a skewed gender distribution among groups, which reached statistical significance ($P = 0.039$). The groups with both infectious agents were composed only by males, while in the group without any agent females were predominant (3:1). The association between sex and infection observed in other studies showed that males were more susceptible than females to EBV and *H pylori* infection[26,27]. Although the gastric antrum was the prevalent site of gastric cancer,

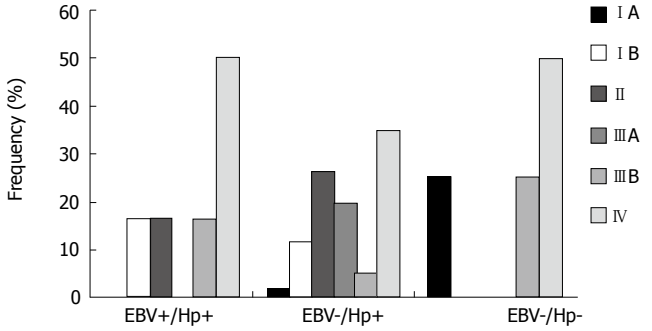


Figure 3 Distribution of the cases by tumor stage in the three defined groups: Hp(+)/EBV(+); Hp(+)/EBV(-); and Hp(-)/EBV(-). $P = 0.179$.

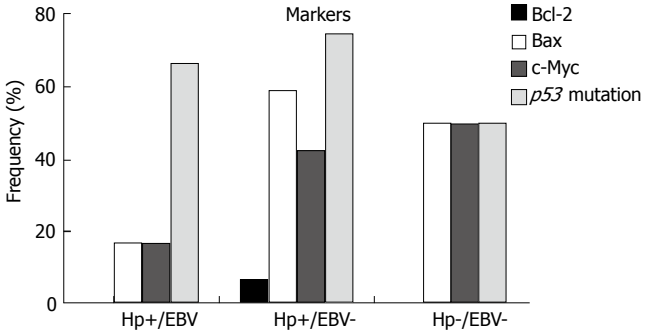


Figure 4 Frequencies of positive cases for the oncoproteins in the three defined groups: Hp(+)/EBV(+); Hp(+)/EBV(-); and Hp(-)/EBV(-). Bcl-2, $P = 0.706$; Bax $P = 0.135$; c-Myc, $P = 0.433$; *p53*, $P = 0.501$.

some studies have shown that EBV is related to the gastric carcinoma in cardia, middle stomach and gastric stump[8]. In our study, we found a slight increase of incidence of tumors located in the cardia in the group with EBV infection compared with patients with *H pylori* infection alone. However, due to the absence of tumors with only EBV infection, we could not evaluate the real relevance of this finding. In spite of the fact that previous studies failed to show a significant association between cardia tumors and *H pylori* infection[6,28], our data point to a possible association between cardia tumors and the presence of either infectious agents, since only the Hp(-)/EBV(-) group did not present tumors located in the cardia[8,29].

The intestinal type was prevalent in all groups, however in the group with *H pylori* infection alone the prevalence of the diffuse and intestinal type were similar, indicating that *H pylori* can be involved with the diffuse type. Reports concerning the association of *H pylori* with histological types of gastric cancers are controversial. Some studies have shown an association between *H pylori* and intestinal type[2,30], while others have observed a balanced distribution between the two histological types, like in the present work[31,32].

Some works have shown an association between Bcl-2 expression and EBV-positive gastric carcinomas[9]. However, the present study did not detect Bcl-2 in the group with EBV infection. On the other hand, Bax was detected in all groups with the highest expression in the group with *H pylori* infection alone, which also presented Bcl-2 expression as mentioned above. Although some authors have shown an increased Bcl-2 expression in *H pylori*-positive cases, in the group (Hp+/EBV-) the Bcl-2 expression was very low[33-37]. Although it was not statistically significant, our findings suggest a possible correlation between *H pylori* and Bcl-2 expression, unlike with EBV.

A few *in vitro* studies have shown that EBV facilitates the tumor development by inducing the expression of the c-Myc protein[39,40]. In present study, the expression of c-Myc was higher in the groups without EBV infection indicating a relationship of c-Myc with gastric carcinogenesis but not with EBV infection[41]. Additionally, some studies point to the involvement of *H pylori* with c-Myc[38,41]. The biological significance between *H pylori* and c-Myc is still not understood. A study from Zang *et al*[38] has linked the c-Myc expression with cell proliferation while a study from Yang *et al*[40] on a gastric carcinoma cell line has pointed to a relationship with apoptosis. On the other hand, Kim *et al*[42] have shown a decrease of c-Myc expression after *H pylori* eradication, but not in its mRNA level, showing the complexity of this process. The data presented here show a high frequency of c-Myc expression in the group with only *H pylori,* but this was similar to the noninfected group. Therefore, the c-Myc expression may be influenced by the presence of *H pylori*, but it is also triggered in a *H pylori* independent way. Nevertheless, it seems that the presence of the EBV may also play a role since the frequency of c-Myc protein was the lowest among the groups, and this may be a disturbing factor when its biologic significance is considered.

The mutation of the *p53* gene is among the major alterations of the multi-step process of gastric carcinogenesis, while it has also been reported in pre-malignant lesions of the stomach, such as chronic gastritis, intestinal metaplasia and dyspepsia. Kodama *et al*[43] have suggested an accumulation of wild-type *p53*, especially in the *H pylori* infected mucosa probably due to the *H pylori*-induced DNA damage. The present study shows the highest frequency of *p53* mutation in the groups with *H pylori* infection, especially among the cagA+ cases, corroborating previous studies[16,3,4]. The high percentage of *p53* mutation in EBV-associated and EBV-negative gastric carcinomas was observed in our study which was also demonstrated in other studies, demonstrating that the *p53* mutation is a relevant alteration in gastric carcinogenesis independent of the infection[15,16,43].

Finally, this study shows the high prevalence of *H pylori* infection in gastric carcinomas in Ceará State. The groups indicate that there are different pathways according to the presence of infectious agents, with female being predominant in the group without infection. *H pylori* seems to influence the expression of Bcl-2, since this protein was observed only in the group infected exclusively by this microorganism. The Bax and c-Myc expression was present in all groups. However, the highest expression in the EBV-negative groups suggests that EBV may inhibit their expression. The mutation of the *p53* gene was present in all groups possibly indicating that it was not only a consequence of the infectious agent.

COMMENTS

Background

Helicobacter pylori and Epstein-Barr virus are important agents on gastric carcinogenesis. Proteins involved with cell cycle or apoptosis process emerge as a candidate for having a crucial role in the development of the neoplasms. Some these, like c-Myc, *p53* and the apoptotic family members such as Bcl-2 and Bax, deserves attention for having a key function in the cell proliferation and cell fate, besides they already have shown involvement in the tumorigenesis process in a variety of tumors, including gastric cancer. So this article tries to associate *H pylori* and EBV infection, at the same time, with gastric carcinoma and some proteins of the cell cycle.

Research frontiers

Helicobacter pylori and Epstein-Barr virus infection related with gastric carcinogenesis.

Innovations and breakthroughs

Although *H pylori* and Epstein-Barr virus were well established as gastric cancer etiological agents, works on gastric cancer never associated an analysis of both agents at the same time, so this article did it.

Applications

Although this work had a small number of cases, it may justify further studies on the issue.

Peer review

This paper demonstrates that *H pylori* infection is very high (94.4%) in Brazil, associated with EBV infection and c-Myc, Bcl-2, Bax and *p53* overexpression in some cases. These results have significant implications on the clinical management and on the research on the pathogenesis of this cancer.

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CLINICAL RESEARCH

Assessment of gastrointestinal stromal tumors with computed tomography following treatment with imatinib mesylate

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Abstract

AIM: To evaluate and characterize the patterns of disease progression of metastatic or unresectable gastrointestinal stromal tumor (GIST) treated with imatinib mesylate, and to determine the prognostic significance associated with disease progression.

METHODS: Clinical data and computed tomography (CT) images were retrospectively reviewed in 17 GIST patients who were treated with imatinib mesylate from October 2002 to October 2006. Apart from using size measurement for evaluation of tumor response [Response Evaluation Criteria in Solid Tumors (RECIST) criteria], patterns of CT changes during treatment were evaluated and correlated with clinical data.

RESULTS: There were eight non-responders and nine responders. Five patterns of CT change during treatment were found: focal progression (FP), generalized progression (GP), generalized cystic change (GC), new cystic lesion (NC) and new solid lesion (NS). At the end of study, all non-responders showed GP, whereas responders showed cystic change (GC and NC) and response according to RECIST criteria. Overall survival was significantly better in patients with cystic change or response within the RECIST criteria compared with GP patients (P = 0.0271).

CONCLUSION: Various patterns of CT change in patients with GIST who responded to imatinib mesylate were demonstrated, and might determine the prognosis of the disease. A combination of RECIST criteria and pattern of CT change are proposed for response evaluation in GIST.

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Key words: Computed tomography; Gastrointestinal stromal tumor; Imatinib mesylate

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are uncommon neoplasms that arise from mesenchymal cells in the walls of the gastrointestinal tract and account for 0.1%-3.0% of all gastrointestinal neoplasms and 5.7% of sarcomas[1,2]. GISTs arise most often from the stomach (60%-70%), followed by small intestine (20%-25%), but rarely from the rectum (5%), esophagus, colon or appendix[3]. GISTs differ from the other mesenchymal neoplasms histopathologically by immunohistochemical expression of CD 117 (c-kit proto-oncogene)[4].

Surgical resection is the standard initial treatment for non-resectable GISTs. However, locally advanced and metastatic tumors of the peritoneum or liver can effectively be treated with imatinib mesylate (known as Gleevec in the United States, Glivec in the rest of the world, and previously referred to as STI 571; Novartis Pharmaceuticals, Basel, Switzerland), a selective-tyrosine kinase inhibitor[5,6]. The drug acts as a c-kit blocker and is highly effective in patients with advanced disease[7], with nearly half of the patients responding to treatment[8].

For optimal clinical outcome, the accurate assessment

of tumor response to imatinib mesylate has become important. Computed tomography (CT), magnetic resonance imaging (MRI), and fluorodeoxyglucose positron emission tomography (FDG-PET) are acceptable imaging techniques to assess response to imatinib mesylate, with FDG-PET being superior[5,9]. CT is commonly used to assess therapeutic response in patients with GISTs. CT enables characterization of primary and recurrent tumors. In the past, the treatment response has been classically quantified using only a decrease in size as the main criterion for tumor response[10]. Recent studies have shown a cyst-like appearance with no further decrease in size is inconsistent with therapeutic response[11-13].

The purpose of this study was to evaluate and characterize the patterns of disease progression of metastatic or unresectable GIST treated with imatinib mesylate. In addition, the study sought to determine the prognostic significance associated with disease progression.

MATERIALS AND METHODS

The study was conducted under the approval of the Institutional Review Board and Ethics Committee of Ramathibodi Hospital Faculty of Medicine, Bangkok, Thailand. Seventeen consecutive patients with metastatic and unresectable GISTs diagnosed histopathologically as positive for CD117 were included in this retrospective review. All patients had a period of imatinib treatment during October 2002 to October 2006. Patients received a starting dose of 400 mg/d imatinib mesylate orally. In the event of disease progression, an increase in daily dosage to 800 mg was allowed. In cases of side effects, the dose was decreased to 300 mg/d. In cases of clinical non-response or death, the treatment was terminated. Our patients included 13 men and four women, aged 36-69 (mean 53) years. The primary tumor sites were the stomach (eight patients), small bowel (six), rectum (two) and retroperitoneum (one patient). At enrollment, 10 patients had initial metastatic disease, three had recurrent disease, three had no free surgical margin, and one had unresectable advanced disease.

Sixty-two lesions (38 in the liver, nine in the mesentery, six in the pararectal region, three in the stomach, two in the peritoneum, two in the lymph nodes, one in the retroperitoneum, and one in bone) were evaluated on the basis of the Response Evaluation Criteria in Solid Tumors (RECIST) criteria for each organ and body compartment invaded by the tumor. Inclusion criteria were as follows: (1) all lesions > 1.0 cm in the longest diameter; and (2) lesions ranging from 0.5 to 1.0 cm in the longest diameter, with no more than five lesions. Exclusion criteria were: (1) lesions < 0.5 cm in the longest diameter; and (2) lesions which were difficult to follow up due to changing location (small bowel and mesentery), or having partial volume.

We retrospectively reviewed the medical records and radiological features of each patient.

Imaging techniques

Each patient underwent baseline CT before treatment (with the exception of patients who underwent scans at another hospital before treatment). Follow-up studies consisted of CT at 1-mo intervals for up to 6 mo after imatinib mesylate treatment. The routine CT at our institution was performed on a spiral CT unit (LightSpeed Plus; General Electric Medical Systems, Milwaukee, WI, USA; Somatom Sensation Cardiac 64; Siemens, USA). CT scans of the upper, lower or whole abdomen were performed (5- or 3-mm slice thickness, 1- or 1.5-pitch, and 5.0- or 7.5-mm collimination) from the level of the diaphragm to the end of the kidney, from the top of the kidney to the pubic symphysis, and from the level of the diaphragm to the pubic symphysis, in the upper, lower and whole abdomen, respectively. Oral contrast, 1000-1500 mL 2% diatrizoate meglumine was given about 2 h prior to scanning, and an intravenous bolus of 100-120 mL 60% non-ionic contrast agent (Omnipaque 300 (GE Healthcare, USA), Ultravist 300 (Schering AG, Germany) or Xentrix 300 (Guerbet, France)) was administered at the rate of 3 mL/s.

A triphasic scanning technique was used after intravenous injection of the contrast agent, with scanning pre-contrast, and delays of 20-30 s and 70-80 s for the arterial and porto-venous phase, respectively.

Image analysis

Images were viewed using the eFilm Workstation software. The CT findings at presentation and during therapy were viewed by one experienced radiologist, who determined the change in size, CT attenuation coefficients, characteristics of tumor images, and overall tumor response. Using eFilm Workstation, tumor size at the longest cross-sectional dimension of each lesion was measured by the same techniques as for baseline measurement. The sum of the longest diameters of lesions in each patient was calculated. The percentage change in the sum of the longest dimensions from the pretreatment evaluation to that at each visit was computed for each patient. The percentage change graded was determined using the RECIST criteria[14].

The CT attenuation co-efficient (density) of the tumor in Hounsfield units (HU) was measured by drawing a region of interest (ROI) that circumscribed the margin of the tumor. The portovenous phase images were used for the tumor-density measurement compared with the pre-contrast phase. The average tumor density was then computed for each patient.

The follow up encompassed all available clinical data, including physical examinations, performance status, laboratory tests, histopathological examinations, and radiology imaging procedures (CT, MRI). For definition of the standard of reference, all patients were rated as responders or non-responders by a medical oncologist at the end of the follow-up. Responders were defined as: (1) improvement or disease-free status that was confirmed by a medical oncologist; and (2) tumor response according to the RECIST criteria (CR, PR and SD). Non-responders were defined as: (1) progression, recurrence, or death from GIST that was confirmed by a medical oncologist; and (2) PD according to the RECIST criteria.

Patterns of CT change after treatment

In addition to distinguishing changes in size using the RECIST criteria, the patterns of changes in CT findings were categorized into five groups. (1) Focal progression

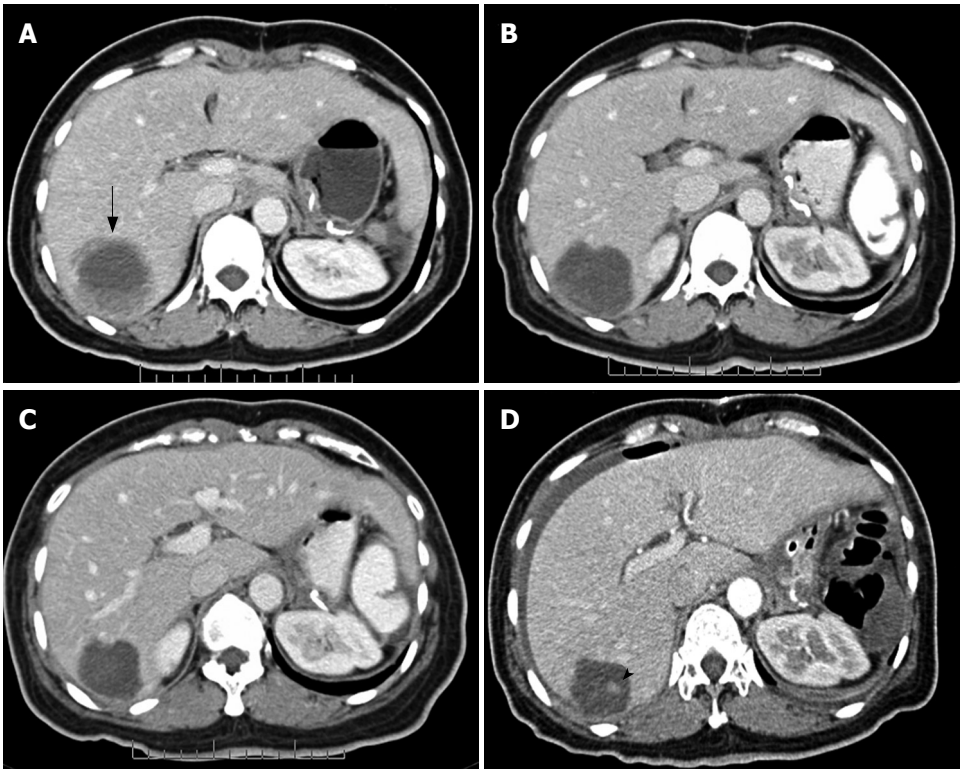


Figure 1 FP pattern. Contrast-enhanced CT of the abdomen obtained from a 39-year-old woman with metastatic GIST in the right lobe of liver. **A**: Before imatinib therapy, the lesion showed ill-defined thickened rim enhancement (arrow); **B**: After 2 mo therapy, a nearly complete cystic change and a thin lesion boundary were observed; **C**: After 5 mo therapy, a further decrease in size (partial response by RECIST) was seen; **D**: After 10 mo therapy, a small enhancing nodule was seen. Nodule within a mass or FP (arrow head).

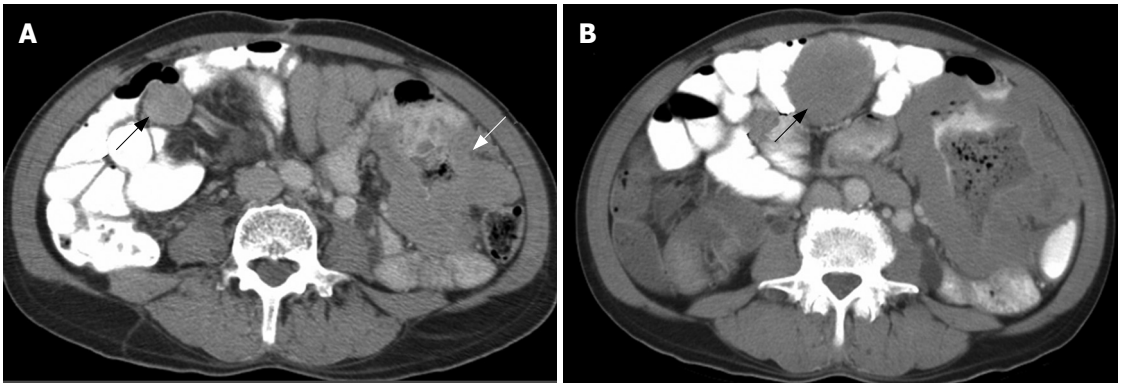


Figure 2 GP pattern. Contrast-enhanced CT of abdomen obtained from a 55-year-old man with metastatic GIST in the mesentery. **A**: Before imatinib treatment, there were mesenteric masses (arrows); **B**: After 2 mo therapy, there was interval progression in both mesenteric masses, as demonstrated by increased tumor size and thickness of the enhancing wall (arrows).

(FP) was defined as single-site progression, which was either an increase in size (PD in the RECIST criteria), an increase in the solid part of the tumor, or development of a new enhancing focus within the pre-existing tumor mass (or described as a nodule within the mass) (Figure 1). (2) Generalized progression (GP) was defined as an increase in tumor size (PD in RECIST criteria), with an enhancing pattern in two or more tumor masses (Figure 2). (3) New solid lesion (NS), with or without a cystic component, was defined as the appearance of one or more new lesions that had an enhancing lesion with or without a cystic component (Figure 3). (4) Generalized cystic change (GC) was defined as the appearance of cystic change in two or more tumor masses (Figure 4). (5) New cystic lesion (NC) was defined as the appearance of one or more new lesions that had relatively low density, without any enhancing component (Figure 5).

Statistical analysis

Statistic analysis was performed with STATA software (StataCorp LP, College Station, TX, USA). Patient age, tumor location, overall response, and pattern of tumor changes were analyzed. Survival was calculated from the day of imatinib treatment until death or the final day of the patient's visit to the outpatient clinic. Kaplan–Meier analysis with a log rank test was used to compare patterns of CT changes and survival distribution. $P < 0.05$ was considered to indicate a significant difference between groups.

RESULTS

Patient characteristics

There were 17 patients included in this study (13 men and four women) with a median age of 52 years (range 36-69 years). Primary tumor sites were in the stomach in eight

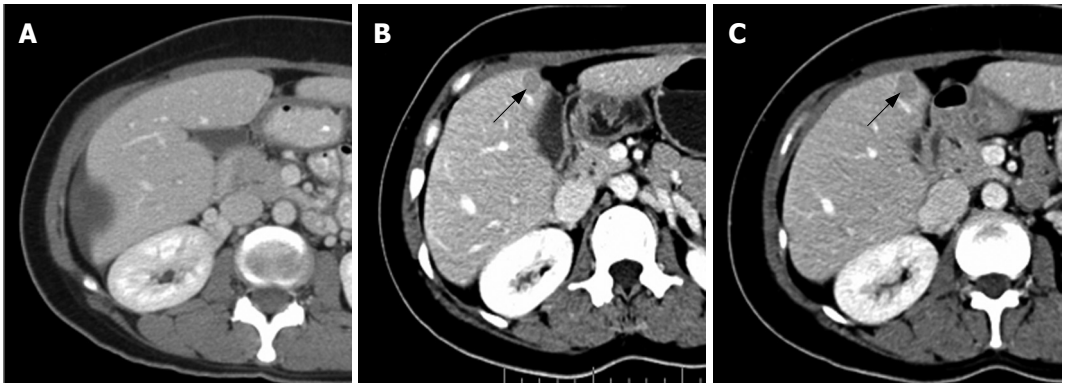


Figure 3 NS pattern. Contrast-enhanced CT scan of abdomen obtained in a 39-year-old woman with metastatic GIST in the liver. **A**: Before imatinib treatment, a large subcapsular cystic lesion in the right hepatic lobe and a small amount of free fluid were noted; **B**: After 10 mo therapy, there were new, well-defined, homogeneous enhancing nodules adjacent to the gallbladder (arrow); **C**: After 13 mo therapy, there was a slight increase in size of the mentioned nodule (arrow).

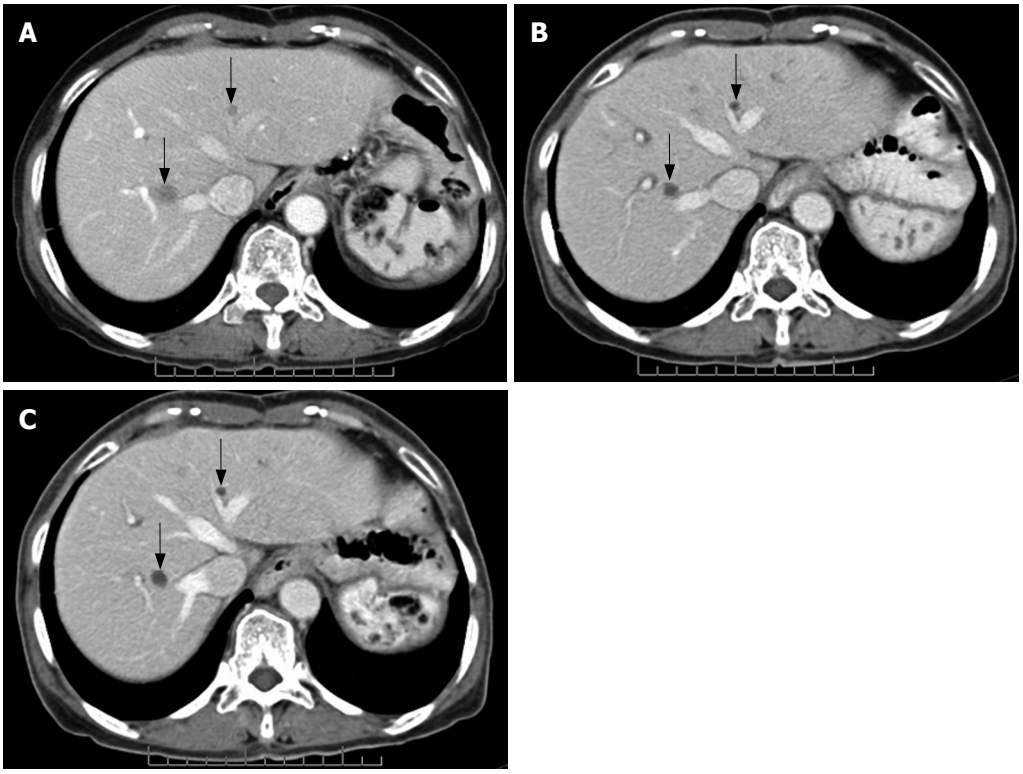


Figure 4 GC pattern. Contrast-enhanced CT scan of the abdomen obtained in a 64-year-old man with metastatic GIST in both lobes of the liver (arrows). **A**: Before imatinib treatment, there were a few rather ill-defined, small homogeneous enhancing lesions in the liver (arrows); **B**: After 4 mo therapy, they showed better-defined, non-enhancing, low-density lesions; **C**: After 8 mo therapy, well-defined small cystic lesions with no interval change in size are noted.

patients (47%), small bowel in six (35%), rectum in two (12%), and retroperitoneum in one (6%). The sites of active disease were in the liver in 10 patients, mesentery in three, pararectal region in three, stomach in three, and one each in the peritoneum, lymph nodes, retroperitoneum and bone. Ten patients (59%) had initial metastatic disease, three (17.5%) had recurrent disease, three (17.5%) had no free surgical margin, and one (6%) had unresectable advanced disease.

Overall response correlating with patterns of CT change

At the end of study, there were eight non-responders and nine responders. Non-responders consisted of two live and six dead patients. The median time of follow up was 11 mo (range 3-29 mo). The majority of active disease was found to be located in the liver, with a minority in the mesentery, retroperitoneum and bone. All patients developed the GP pattern at the end of follow up. Two patients developed FP, whereas one developed NS. One of the FP patients showed initial GC and NC. For one patient who had active sites in the liver and mesentery, GP was observed in the mesentery, while the liver lesions showed a GC pattern. The remainder of GP and NS had the same pattern throughout till the end. The median time to GP was 5.5 mo (range 2-23 mo). However, one patient showed GP after a prolonged 23 mo of SD and had early initial NC.

There were nine responders. They showed GC, NC, and response according to RECIST criteria in five, one and three patients at the end of study, respectively. The active sites of GC were in the liver (one patient), pararectum (two), and one each in the stomach and peritoneum/intra-abdominal nodes. One patient showed NC in the liver. The median time to GC and NC was 3.5 mo (range 2-8 mo). Of the three patients with no change in pattern, two

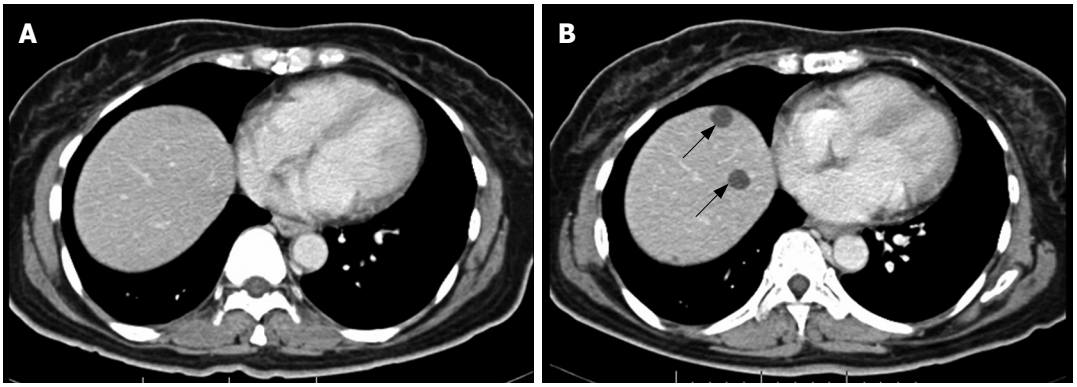


Figure 5 NC pattern. Contrast-enhanced CT scan of abdomen in 50-year-old woman with metastatic GIST in the liver. **A**: Before imatinib treatment, no liver metastases were visible at this level; **B**: Contrast-enhanced CT after 2 mo treatment with imatinib. At least two non-enhancing low-density lesions were newly seen at the dome of the liver (arrows).

Table 1 Median survival time according to pattern of CT change

Patterns of CT change	No. of patients (%)	No. of deaths	Median survival time (mo)	*P* value
GC + NC	6 (35)	0	-	0.00271
CR + PR + SD	3 (18)	0	-	0.00271
GP	8 (47)	6	11	0.00271

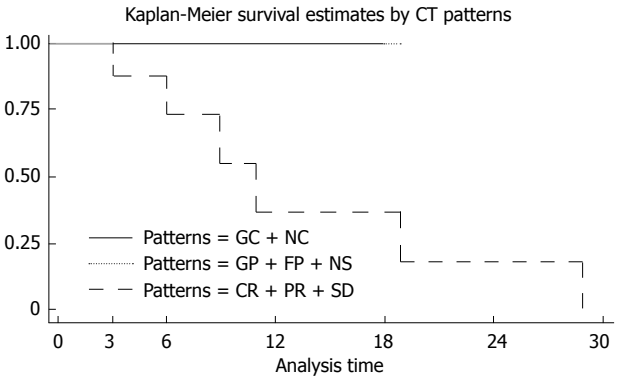


Figure 6 Overall survival after imatinib therapy, according to the patterns of CT change.

patients had a change in size only (PR and CR within the RECIST criteria), while the third patient showed a slight decrease in size (SD). The patient with CR had an active site in the stomach (no free margin in surgery) without metastasis, and the time to CR was 4 mo. The SD patient had an active site in the mesentery. Half of the responders showed PR in the initial follow up.

Survival data analysis according to patterns of CT change

Complete information on the patients' clinical course of imatinib treatment was obtained in 17 cases. Median follow-up time was 15 mo (range 3-29 mo). Survival status was alive in 11 (64.7%) and dead due to disease in six (35.3%). Median overall survival time of the patients after imatinib treatment was 19 mo (95% CI, 9.3-80.5). Overall survival was better in GC/NC and CR/PR/SD (response according to RECIST) compared with GP patients, and *P* was 0.0271 (Table 1, Figure 6).

DISCUSSION

GIST is an uncommon tumor with no effective therapy in advanced disease. This has been the case for inoperable tumors until the advent of imatinib mesylate, which is a molecular targeted therapy. Imatinib mesylate is the first effective systemic treatment for advanced GISTs, and yields a benefit of 50%-80%[15]. Although, the efficacy and safety of imatinib mesylate have been examined in large studies, few have focused on the pattern of tumor changes after treatment. Knowledge on these patterns of response to treatment are important to adequately manage patients and to interpret clinical trials employing new tyrosine kinase inhibitors known as sunitinib[16].

The percentage overall response with imatinib treatment in this study was nearly equal to that in a previous study, which showed an overall response rate of about 50%, with 5% of those with a CR demonstrating a clinical response by CT scan[8].

This study demonstrated different patterns of CT changes in responders and non-responders during treatment with imatinib mesylate. Apart from the RECIST criteria, cystic change (GC, NC) was used to evaluate the response to treatment. If the lesions showed cystic change, even though it was a new lesion or a cystic change in a previous lesion, it was considered to be disease improvement. Several studies have supported the finding that cystic change is a feature of tumors which have responded to treatment, due to tumor necrosis and cystic or myxoid degeneration[10,17-19]. Many authors have suggested new cystic lesions are characteristic of a response in small solid hepatic lesions, which cannot be seen in the initial image due to iso-density compared to liver parenchyma[20-22]. FDG-PET in these patients has confirmed no glucose radiotracer uptake in the cystic lesions, whereas the tumor size remains unaltered[8,23,24]. This suggested there were no metabolically active tumor cells.

The non-responder lesions showed three patterns of disease progression during treatment: GP, FP and NS. These patterns represented an increased solid component in terms of generalized or focal change. The FP pattern manifested as an increase in the solid component, such as increased wall thickness or a nodule within a mass[25,26]. Several studies have demonstrated that a solid nodule appearing inside a residual cystic mass indicating early retrogression after partial response to imatinib mesylate that corresponded to depicting new foci of increased FDG

uptake in PET scan[11,23]. Few non-responders showed early FP or NS before developing GP, and finally, death. This may urge further treatment, such as surgery of the focal mass, or the new tyrosine kinase inhibitor sunitinib[16,27-30]. Additionally, one of non-responders showed relapse of FP after cystic change of hepatic lesions. The authors speculated this event may have been a regrowth from a residual tumor cell. However, the patient had prolonged stable disease before developing GP.

Interestingly, there was one non-responder, who had two active sites of disease and showed a mixed response. The patient had GC in hepatic lesions but GP in mesenteric lesions. Thus, evaluation of the tumor response should be analyzed on a lesion by lesion basis.

The present study had several limitations. First, a small number of patients were enrolled. Second, the follow-up duration after imatinib treatment was uneven (range 1-6 mo), due to lack of certain protocol criteria for imaging follow up. Third, there was no gold standard reference (such as surgical pathology after imatinib treatment or FDG-PET scan) for evaluation of tumor response.

In conclusion, various patterns of CT changes for evaluation the tumor response were presented which might determine disease prognosis. A combination of RECIST criteria and patterns of CT change have been proposed for better response evaluation in GISTs. The early detection of focal solid or new solid lesions after maximal dose of imatinib mesylate suggests disease progression and might be helpful in early intervention such as surgery or new tyrosine kinase inhibitors.

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COMMENTS

Background

For optimal clinical outcome, the accurate assessment of tumor response to any treatments is important. RECIST criteria have commonly been used to assess response in solid tumors for decades. The criteria are based solely on changes in size of mass on cross-sectional imaging studies. However, there are circumstances in which the response according to RECIST criteria does not correspond to clinical outcome. This means other imaging criteria may be needed to improve assessment. There are observations that show changes in pattern enhancement can be different in tumors after treatment. Among solid tumors, metastatic GISTs are a good example to show how pattern of enhancement in CT can predict clinical outcome.

Research frontiers

Many new anticancer drugs are being developed, particularly those which affect tumor blood vessels, so-called antiangiogenic drugs. Imatinib mesylate (also known as Gleevec, a selective-tyrosine kinase inhibitor) is one of these, and is highly effective in patients with metastatic GISTs. Due to its effect on tumor angiogenesis, the change may be able to be observed during dynamic contrast enhancement acquisition by using CT or MRI techniques.

Innovations and breakthroughs

This study showed evaluating response of metastatic GISTs to imatinib by size alone is not enough. Combined changes in density or pattern enhancement on CT are more reliable.

Applications

The combined use RECIST criteria and changes in the pattern of enhancement are useful in evaluating tumor response, particularly in patients treated with antiangiogenic drugs or other means that have an effect on tumor blood vessels.

Peer review

This is an interesting study on the follow-up of non-resectable GIST treated with imatinib. Tumor response was assessed by the RECIST criteria complemented by other criteria on CT. Overall, the results show beautiful iconography.

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RAPID COMMUNICATION

Protective effect of some vitamins against the toxic action of ethanol on liver regeneration induced by partial hepatectomy in rats

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Abstract

AIM: To investigate the effects of vitamins (A, C and E) on liver injury induced by ethanol administration during liver regeneration in rats.

METHODS: Male Wistar rats subjected to 70% partial hepatectomy were divided into five groups (groups 1-5). During the experiment, animals of Group 1 drank only water. The other four groups (2-5) drank 30 mL of ethanol/L of water. Group 3 additionally received vitamin A, those of group 4 vitamin C and those of group 5 received vitamin E. Subsequently serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin and bilirubin were measured colorimetrically. Lipid peroxidation (thiobarbituric-acid reactive substances, TBARS) both in plasma and liver was measured, as well as liver mass gain assessment and total DNA.

RESULTS: Compared with sham group, serum AST and ALT increased significantly under ethanol treatment (43% and 93%, respectively, with $P < 0.05$). Vitamin C and vitamin E treatment attenuated the ethanol-induced increases in ALT and AST activity. Ethanol treatment also decreased serum albumin concentration compared to sham group (3.1 ± 0.4 g/dL *vs* 4.5 ± 0.2 g/dL; $P < 0.05$). During liver regeneration vitamins C and E significantly ameliorated liver injury for ethanol administration in hepatic lipid peroxidation (4.92 nmol/mg and 4.25 nmol/mg *vs* 14.78 nmol/mg, respectively, with $P < 0.05$). In association with hepatic injury, ethanol administration caused a significant increase in both hepatic and plasma lipid peroxidation. Vitamins (C and E) treatment attenuated hepatic and plasma lipid peroxidation.

CONCLUSION: Vitamins C and E protect against liver injury and dysfunction, attenuate lipid peroxidation, and thus appear to be significantly more effective than vitamin A against ethanol-mediated toxic effects during liver regeneration.

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Key words: Lipid peroxidation; Antioxidant vitamins; Alcohol-induced liver injury; Liver regeneration

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INTRODUCTION

Alcohol liver disease is a world-wide medical complication of alcohol abuse[1]. Although important progress has been made in understanding its pathogenesis, the mechanisms involved in its development are as yet not fully understood.

Oxidative stress is a key step in the pathogenesis of ethanol-associated liver injury. The deleterious effects of alcohol, at least partly involve oxidative injury that has been documented by measurement of oxidant radicals, alterations in oxidant/antioxidant balance and oxidant induced changes in cellular proteins and lipids[2].

After ethanol is absorbed, it is distributed to all tissue, and body fluids in direct proportion to blood levels[3]. Ethanol affects the liver more than any other organ[4], since its metabolism implies generation of free radicals that can damage cell structure and function, particularly when antioxidant mechanisms are not able to neutralize them[5,6]. Animal models show the mechanisms by which both acute alcohol administration and chronic alcohol consumption affect the liver. Chronic ethanol administration induces an increase in lipid peroxidation either by enhancing the production of oxygen reactive species, or by decreasing the level of endogenous antioxidants[7]. Short-term ethanol administration has been reported to decrease vitamins and increase superoxide generation in the liver[8]. The enhanced superoxide generation increases lipid peroxidation and induces liver dysfunction in rats that have been subjected to acute ethanol exposure[9].

One of the most prominent antioxidant defense systems are vitamins (A, C and E) acting as co-factors for many enzymes, protecting cells against free radical-mediated damage[10], as free radical scavengers. Besides, large ethanol intakes have deleterious effects on nutritional status[7,8].

Recently, it has become clear that antioxidant nutrients, including vitamins, are important for neurological function[11-13]. High intake of vitamins E and C have been found to be associated with lower risk of Alzheimer's disease[14]. In addition, evidence for alcohol-induced oxidant injury comes from studies in which pretreatment with antioxidants such as vitamin E, vitamin C, and agents that enhance antioxidant capacity attenuate alcohol induced effects[15].

Nutrient intermediary metabolism is a vital function of the healthy adult liver. Fortunately, the liver appears to have a tremendous reserve capacity in this regard, since general nutritional status is well-preserved until severe hepatic parenchymal loss occurs. Indeed, several clinical and biochemical markers of nutritional status are often used to gauge the severity of liver dysfunction, with evidence of severe malnutrition implying end-stage liver disease. Hepatocyte proliferation occurs rarely in healthy adults, but it is necessary to replace liver cells lost by hepatic necrosis or resection. These facts have prompted speculation on whether decreased hepatic mass and metabolism are involved in the initiation and progression of hepatic regeneration following liver injury[16]. Restoration of normal hepatic structure and function is the goal of therapy for acute and chronic liver injury of varying etiologies. Consequently, strategies to optimize compensatory hepatic regenerative growth have tremendous therapeutic relevance. Better understanding of the mechanism regulating cellular proliferation, and organ regeneration will play a critical role in designing treatments to enhance liver regeneration[16]. Current knowledge suggests several approaches. Thus, novel agents that correct the fundamental cellular disturbances resulting from excessive ethanol consumption represent attractive therapeutic strategies.

In the present study, we investigated the effects of vitamins A, C and E in rats with liver injury induced by ethanol administration during liver regeneration. Specifically, we planed to evaluate: (a) whether the effect of the vitamin supplementation protects against ethanol-induced lipid peroxidation; (b) whether vitamin supplementation can attenuate ethanol-induced hepatic injury and dysfunction during liver regeneration.

MATERIALS AND METHODS

Animal and chemicals

Male Wistar rats (230-270 g, body weight) were obtained from Centro Médico Nacional "20 de Noviembre" ISSSTE-MEXICO. The rats were housed in animal quarters at the Escuela de Dietética y Nutrición ISSSTE-MEXICO. They were maintained at 22℃ with a 12-h light/dark cycle and allowed to consume standard rat pellet chow (Purina de México SA) and water *ad libitum* before treatments. After 10 d of adaptation the procedure was begun. All procedures involving experimental animals were done according to our Federal Regulations for Animal Experimentation and Care (Ministry of Agriculture; SAGAR, Mexico).

Vitamins A, C and E and thiobarbituric acid were from Sigma (Sigma Chemical St. Louis MO). All other chemicals were from the best quality available.

Surgical procedures

Two-thirds partial hepatectomy (PH) was performed according to the technique of Higgins and Anderson[17]. The operations were carried out between 08:00 and 10:00 hours under light diethyl ether anesthesia and consisted of removal of the median and left lateral lobes of the liver. As controls, sham-operated rats were subjected to the same surgical procedure, without remission of the liver mass.

Experimental designs

After surgery, rats were housed individually and fed with rat pellet chow. They were grouped (n = 30 for each experimental group) as follows: (1) control rats (sham operated and PH-only treated rats) who received water *ad libitum*; (2) PH rats who received a 30 mL/L ethanol solution in their drink container (PH-ethanol group) in a voluntary way, the intake being registered daily. Ethanol consumption was calculated in terms of g/kg per day. Ethanol consumption usually averaged 1.5 ± 0.5 g/kg per day and blood alcohol levels have been reported to vary between 75 mg/dL and 150 mg/dL[18-20]. On the other hand, PH-ethanol treated rats were divided into the following treatment groups: (a) PH-ethanol treated rats with intraperitoneal doses of vitamin A (PH-ethanol-Vitamin A group), 4000 UI per day[21]; (b) PH-ethanol treated rats with intragastric doses of vitamin C (PH-ethanol-Vitamin C group), 250 mg/kg per day[22]; and (c) PH-ethanol treated rats with intraperitoneal doses of vitamin E (PH-ethanol-Vitamin E

group), 250 mg/kg per day[22]. All the treatments (ethanol solution and vitamins) were given daily during seven days; the animals' weights were recorded daily.

Serum and liver samples

On the eighth day of treatment, animals were killed by decapitation previously anesthetized with sodium pentobarbital (40 mg/kg of b.w.). Blood samples were obtained and serum isolated. Serum was kept frozen at -70℃ until use. The liver was isolated and weighed and it was placed quickly in an ice-cold PBS (phosphate saline buffer solution, pH 7.5) and washed until it was bloodless. The liver was placed in 9 volumes of ice-cold solution Tris-HCL buffer (50 mmol/L Tris-HCl, 180 mmol/L KCL, 10 mmol/L EDTA, and 0.02% butylated hydroxytoluene; pH 7.4) and homogenized using four to five strokes of a Teflon homogenizer spinning at 1000 r/min. Homogenate was divided into samples and frozen at -70℃ until its subsequent use. Total protein concentration was determined by the Lowry method[23], using bovine serum albumin (BSA) as a standard.

Enzymes and metabolites assay from serum

Serum enzymes, alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) activities as indicators of the extent of liver injury were measured colorimetrically using a diagnostic kit (Sigma Chemical Co., St. Louis, MO) following the instructions provided by the manufacturers.

Serum albumin and total bilirubin concentrations, documenting the extent of liver dysfunction, were determined colorimetrically using a Sigma Diagnostic kit (Sigma Chemical Co., St. Louis, MO) following the instructions provided by the manufacturer.

Thiobarbituric acid reactive substances assay

To determine the thiobarbituric acid reactive substances (TBARS) in liver, homogenate was used according to method of Song[24]. Briefly, to 0.2 mL of the tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid, 1.5 mL of 0.9% thiobarbituric acid, and 0.6 mL of distilled water were added and vortexed. The reaction mixture was placed in a water bath at 95℃ for 1 h. After cooling on ice, 1.0 mL of distilled water and 5.0 mL of butanol/pyridine mixture (15:1, v/v), were added and vortexed. After centrifugation at 10000 r/min for 10 min, the resulting upper phase was determined at 532 nm. The concentration of TBARS was calculated using 1, 1, 5, 3-tetraethoxypropane as a standard[24]. In the serum, the determination of TBARS concentration was made as described above with 200 μL serum.

Restitution of liver weight and total DNA measured

Hepatic regeneration was determined by the calculation of liver weight restitution and total DNA concentration. The extent to which the liver mass had been reconstituted was also evaluated. Resected livers were weighed at the time of PH. This weight was divided by 0.7 to derive the initial (pre-PH) weight of each liver. Each post-PH liver remnant was also weighed at the time of death. For each rat, the weight of the remnant liver was normalized to the weight of that rat's entire liver at the time of PH and expressed as the percentage of the rat's initial liver weight according to the following formula: (wt of liver remnant/initial liver wt) × 100[25]. Data from all PH rats per treatment group were used to calculate the mean ± SE at the end of the experimental time. DNA concentrations were determined fluorometrically according to Labarca and Paigen[26].

Table 1 Mortality and body weight parameters in each experimental group after seven days of treatment partial hepatectomy (PH), ethanol (EtOH) and some vitamins

Group	Mortality (%)	Initial body weight[1] (g)	Final body weight[1] (g)	Differential between final and initial weight (g)
Sham	0/30 (0)	259 ± 10	274 ± 1	15
PH	0/30 (0)	250 ± 11	267 ± 10	17
PH-EtOH	22/30 (73.3)[a]	255 ± 10	276 ± 15	21
PH-EtOH-VA	16/30 (53.3)[e]	255 ± 12	270 ± 12	15
PH-EtOH-VC	10/30 (33.3)[e]	260 ± 12	275 ± 10	15
PH-EtOH-VE	8/30 (26.6)[e]	242 ± 10	258 ± 11	16

[1]Values are the mean ± SE of survivors for each experimental group (n = 8-22) as it corresponds. VA: Vitamin A; VC: Vitamin C; VE: Vitamin E. [a]$P < 0.05$ *vs* the sham group; [e]$P < 0.05$ *vs* hepatectomized + ethanol group.

Statistical analysis

All data are expressed as mean ± SE. Statistical analysis was performed using Student's t-test and ANOVA where appropriate. Differences between groups were considered to be statistically significant at $P < 0.05$.

RESULTS

Mortality and body weight

The effect of the combination of ethanol and vitamins on the mortality and the body weight of the rats is shown in Table 1. It is observed that the ethanol group (PH-EtOH), presented a significant mortality (73.3%; $P < 0.05$) in comparison with the sham group and the group the PH-only group. On the contrary, the administration of the vitamins A, E and C in the ethanol group, decreased mortality, increasing significantly ($P < 0.05$) survival capacity as compared with the PH-EtOH group; 73.4% for the vitamin E group (PH-EtOH-VE), 66.7% for the vitamin C group (PH-EtOH-VC) and 46.7% for the vitamin A group (PH-EtOH-VA). As for the body weight, there were differences observed in the initial weight (after the hepatectomy) and the final weight (seven days after treatment) in the animals of all the experimental groups, which confirms that this parameter is not altered by the surgery or other used substances.

Ethanol and vitamins effect over the liver regeneration

Table 2 shows the results of the indicators (restitution of the liver mass, liver/body ratio and mgDNA/g liver ratio) associated with the process of hepatic regeneration. The PH-EtOH group had a significant decrease in the hepatic indicators compared to the PH-only group. Except for the PH-EtOH group, all the experimental groups treated with

Table 2 Evaluation of liver regeneration parameters in each experimental group after seven days of treatment with partial hepatectomy (PH), ethanol (EtOH) and vitamins. Experimental designs are described in material and methods section

Group	Restitution liver mass[1] (%)	Resected liver mass[1] (g)	Final liver weight[1] (g)	Liver/body ratio[1] (%)	mgDNA/g liver[1]
Sham	None	None	9.41 ± 0.3	3.41 ± 0.2	2.1 ± 0.2
PH	83.95 ± 3.0	6.9 ± 0.3	8.27 ± 0.3	3.09 ± 0.3[a]	4.1 ± 0.4[a]
PH-EtOH	54.89 ± 2.0[c]	6.8 ± 0.4	5.33 ± 0.2[c]	1.93 ± 0.1[a,c]	2.9 ± 0.1[a,c]
PH-EtOH VA	62.90 ± 3.9[c,e]	7.1 ± 0.3	6.38 ± 0.4[c]	2.36 ± 0.3[a,c,e]	3.4 ± 0.2[a,c,e]
PH-EtOH-VC	79.00 ± 2.0[c]	7.0 ± 0.5	7.90 ± 0.2[e]	2.87 ± 0.2[a,e]	4.0 ± 0.3[a,e]
PH-EtOH-VE	81.00 ± 3.0[c]	6.8 ± 0.2	7.86 ± 0.3[e]	3.03 ± 0.3[a,e]	3.9 ± 0.4[a,e]

[1]Values are the mean ± SE of survivors for each experimental group (n = 8-22) as it corresponds. Vitamin A (VA); Vitamin C (VC); Vitamin E (VE). [a]$P < 0.05$ *vs* the sham group; [c]$P < 0.05$ *vs* the hepatectomized group; [e]$P < 0.05$ *vs* hepatectomized + ethanol group.

Table 3 Albumin and bilirubin levels on serum from rats after seven days of partial hepatectomy (PH) and daily ingestion of ethanol and application of vitamins A, C or E

Group	Albumin[1] (g/dL)	Bilirubin[1] (mg/dL)
Sham	4.5 ± 0.2	0.42 ± 0.01
PH	4.7 ± 0.3	0.40 ± 0.02
PH-EtOH	3.1 ± 0.4[a,c]	1.72 ± 0.04[a,c]
PH-EtOH VA	3.5 ± 0.3[a,c]	1.91 ± 0.06[a,c]
PH-EtOH-VC	4.2 ± 0.2[e]	0.45 ± 0.01[e]
PH-EtOH-VE	4.6 ± 0.3[e]	0.29 ± 0.03[a,c,e]

[1]Values are the mean ± SE of survivors for each experimental group (n = 8-22) as it corresponds. vitamin A (VA); Vitamin C (VC); vitamin E (VE). [a]$P < 0.05$ *vs* the sham group; [c]$P < 0.05$ *vs* the hepatectomized group; [e]$P < 0.05$ *vs* hepatectomized + ethanol group.

ethanol in combination with the vitamins showed a recovery in the hepatic regeneration process; but the group that obtained the major protection was the one treated with vitamin E (PH-EtOH-VE) and vitamin C (PH-EtOH-VC), obtaining values comparable to the PH-only group.

The difference with the PH-only group with the vitamin E group (PH-ETOH-VE) and the vitamin C group (PH-EtOH-VC) was 2.95% and 4.95% for the restitution of the liver mass, 0.06% and 0.22% for the liver/body ratio, and 0.2 and 0.1 of mgDNA/g of liver, respectively. On the other hand, the group treated with ethanol and vitamin A (PH-EtOH-VA) only had a recuperation of 62.9% for the restitution of the liver mass, being significantly different from the PH-only group (83.9%, $P < 0.05$), and 2.36% (*vs* 3.09%, $P < 0.05$) and 3.4% (*vs* 4.1%, $P < 0.05$) for the other parameters, liver/body ratio and mgDNA/g of liver, respectively.

Effects of vitamin treatment on serum metabolite levels

The results of the liver metabolic integrity are presented in Table 3. There are similar concentrations of serum (bilirubin and albumin) both in animals with hepatectomy as well as the sham group animals. When ethanol was administered as in the (PH-EtOH) group, there was a significant decrease in serum albumin (31 g/L *vs* 47 g/L, $P < 0.05$) and an increase in bilirubin (17.2 mg/L *vs* 4.0 mg/L, $P < 0.05$) compared to the PH-only group. This serum alteration is normalized when ethanol and one of the vitamins is administered in combination, giving a better recuperation of both metabolites with vitamin C or E, since albumin increases its concentration to 42 g/L in the PH-EtOH-VC group and 46 g/L in the PH-EtOH-VE group, while in bilirubin their values are reduced to 4.5 mg/L and 2.9 mg/L respectively. The group treated with vitamin A did not reach values comparable to the PH-only group because its behavior was similar to the PH-EtOH group.

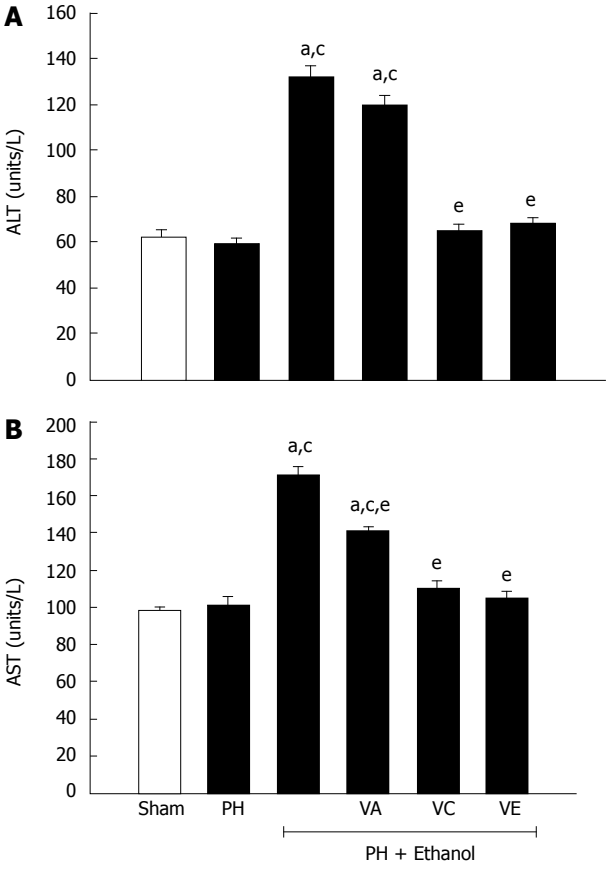


Figure 1 Effect of vitamins on specific activities of serum alanine and aspartate aminotransferase (**A** and **B**) during liver regeneration and ethanol intoxication. Values are means ± SE (n = 8-10); [a]$P < 0.05$ *vs* the sham group (sham); [c]$P < 0.05$ vs the hepatectomized group (PH); [e]$P < 0.05$ *vs* hepatectomized + ethanol group (PH + Ethanol). VA: Vitamin A; VC: Vitamin C; VE: Vitamin E.

Effects of vitamin treatment on serum enzymes activities

The protective effect of the vitamins was evaluated with the enzymatic activity of the alanine aminotransferase (ALT) and the aspartate aminotransferase (AST) because they reflect the liver function. As is shown in Figure 1, the ethanol increased significantly the serum concentration of both enzymes, observing an activity of 2-times for ALT (A)

and 1.7-time for AST (B) compared to PH-only and sham groups. A similar effect is observed in the animals treated with ethanol plus the vitamin A, giving evidence of the low potential of this vitamin to promote hepatic regeneration. On the contrary, the partial hepatectomized rats treated with combinations of ethanol and vitamins C or E showed a significant decrease in the serum concentration of ALT and AST levels, reaching values comparable to the control group (sham group).

Effect of vitamins on TBARS-induced by ethanol treatment in PH rats

To evaluate the damage produced by the reactive oxygen species (ROS), the TBARS concentration was determined in liver and serum in the animals treated with ethanol and the diverse vitamins (Figure 2). An increase of almost double is observed in the hepatic concentrations of TBARS (panel A) in the rats with PH in comparison with the sham group. This increase becomes more evident, when the animals are treated for seven days with ethanol, with 14.78 nmol/mg, which is 3.3 times and 1.4 times greater than the sham group (4.48 nmol/mg) and the PH-only group (10.55 nmol/mg), respectively.

Contrary, the three groups with ethanol and a vitamin showed a decrease in the hepatic concentration of TBARS, although the most relevant protection was observed in the PH-EtOH-VC (4.92 nmol/mg) and PH-EtOH-VE group (4.25 nmol/mg), since these values were similar to the sham group (4.48 nmol/mg). It is important to mention, that the PH-EtOH-VA group (10.74 nmol/mg) only presented a reduction comparable to the PH-only group (10.55 nmol/mg).

In the case of the corresponding concentrations in serum (Figure 2, panel B), the rats of the PH-EtOH group presented a significant increase of 80% in relation to the sham group. On the contrary, when vitamin C or E was administrated to the rats intoxicated with ethanol, there were no differences observed in the serum concentrations of TBARS in comparison with the rats of the PH-only group. Contrary, when vitamin A was administered, the serum TBARS group was similar to the PH-EtOH group, observing an increment of 20% and 22% with respect to the PH-EtOH-VC and the PH-EtOH-VE groups respectively.

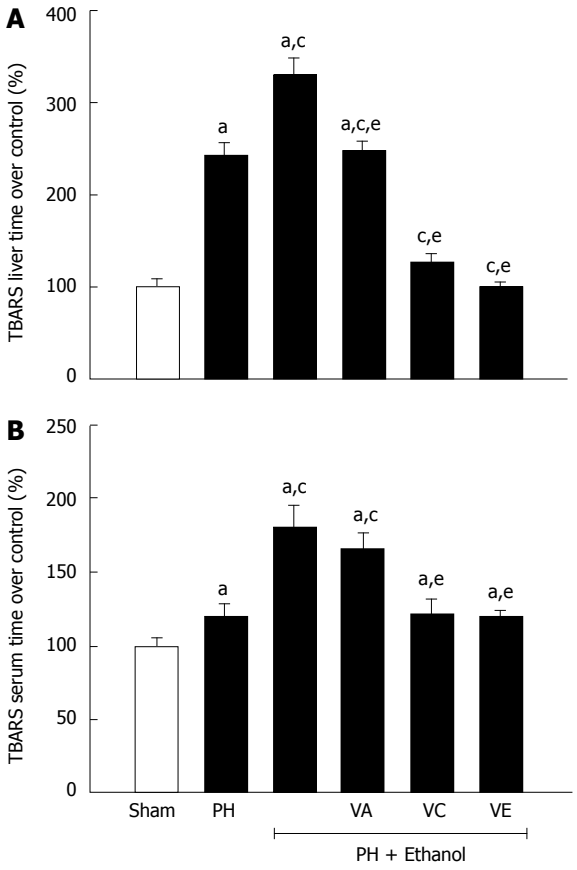


Figure 2 Effect of vitamins in both hepatic (**A**) and serum (**B**) lipid peroxidation (TBARS content) during liver regeneration and ethanol intoxication. Values are means ± SE (n = 8-10); $^{a}P < 0.05$ *vs* the sham group (sham); $^{c}P < 0.05$ *vs* the hepatectomized group (PH); $^{e}P < 0.05$ *vs* hepatectomized + ethanol group (PH + Ethanol). VA: Vitamin A; VC: Vitamin C; VE: Vitamin E.

DISCUSSION

Epidemiologic studies agree about the existing correlation between the ingestion of alcohol and the high incidence of hepatic cancer, but the exact mechanism of the hepatocarcinogenesis is still a ground research because ethanol by itself is not a carcinogenic compound[27-32]. Besides several mechanisms involved in the development of a hepatocarcinogenic process which have been identified, the formation of acetaldehyde through the oxidation of ethanol by the action of the cytochrome P-4502E1 (CYP2E1) continues to be the most accepted one because of the production of reactive oxygen species (ROS) during the biotransformation, which consequently can alter cellular regeneration[33].

It has been observed that the chronic consumption of alcohol increases the activity of CYP2E1. This enzyme has been related to the lipoperoxidation process and the excessive generation of hydroxyl ethyl radicals, resulting in a decrease in endogenic antioxidants' levels[34]. In other words, the oxidative stress produced by ethanol is critical in post alcoholic hepatogenesis due to the production of free radicals which damage the cellular structure and function, especially if natural antioxidant mechanisms cannot neutralize them[28,33].

These observations support the idea that pre-treatment with substances that reduce the activity of CYP2E1 or that inactivate the ROS, could reduce the hepatotoxic effects of ethanol. Following this hypothesis, the objective of this study was to evaluate the antioxidant potential of three vitamins A, C, and E against the hepatic damage produced by the sub chronic administration of ethanol during the cellular regeneration after a partial hepatectomy (PH).

Initially, it was observed that ethanol (1.5 g/kg) increased mortality, which supports the toxicity of ethanol when it is given in a subchronic way; confirming its capacity to produce different alterations in the organism, like nuclear condensation, cellular edema with abundant accumulation of fat, and an abnormal mitochondrial activity[35].

On the other hand, when ethanol was given in combination with the different vitamins, the survival

percentage increased (Table 1), supporting the evidence of the importance of these compounds in the adequate development of an organism and backs up the studies carried out by different researchers, showing the capacity of vitamins to interact with free radicals decreasing the toxicity to the organism[10,15]. Normally, the excessive and constant ingestion of ethanol produces a weight decrease in the individuals because alcohol modifies several metabolic pathways and at the same time, displaces an adequate diet for the necessity of alcohol consumption, but the opposite was observed in this experiment (Table 1), which may be explained by inadequate doses of ethanol or the insufficient experimentation time to be able to produce the dependency to this substance[15,36].

In relation to the ethanol effect over the liver mass, the liver/body ratio and the mgDNA/g liver (Table 2), the data of the present work gives evidence of the utility of these indicators in the evaluation of the cellular regeneration showing that the given doses of ethanol (1.5 g/kg) decreases these parameters significantly. This agrees with the study of Yoshida[37], where an immunoblotting assay was used to evaluate the same dose of ethanol over cellular regeneration that altered PH and determined that alcohol attenuated the hepatic activity of the thymidylate synthase and the thymidine kinase at 24 h altering surgery, and affecting the quantity of DNA as well. The Northern blot analysis confirmed that the protein levels of the enzymes are proportional to the quantity of mRNA, so ethanol is capable of inhibiting the synthesis of DNA by reducing the mRNA levels of these enzymes during hepatic regeneration[37].

The previous study and this one agrees with the suggestions of other authors to consider the PH as the good *in vivo* model most adequate to study cellular proliferation because it is a relatively simple technique and with the advantage that after surgery, only a short time is required to evaluate hepatic regeneration. Besides, with the quantification of liver mass, liver/body ratio and mgDNA/g liver (Table 2) hepatic regeneration is evaluated indirectly; it has been observed that it depends on the integrity of the mitochondrial respiration mechanism and the cellular capacity of ATP formation, the proper formation of proteins and therefore the synthesis of mRNA and DNA[38-40]. Unlike most other terminally differentiated cells, the hepatocytes in adult liver retain the capacity to proliferate. Indeed, following 70% partial hepatectomy, most hepatocytes proliferative at least once. Hepatocellular proliferation continues until the prehepatectomy liver mass is restored. This compensatory hyperplasia being within minutes of resection is typically completed within 2 wk in rats and in less than one month in humans[17].

Since the 80's the equilibrium between the generation of free radicals and the endogenous antioxidant mechanisms have been recognized. It has been established that this balance can be altered during oxidative stress induced by different xenobiotics and it can also be reestablished when antioxidant compounds like ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), or β-carotene (vitamin A) are administrated[41,42]. The results of this study (Figure 2) support this observation, since the 3 vitamins showed a protective effect against the generation of hydroxyethyl radicals generated by the ethanol; although the most significant efficiency was with vitamin C and E, where the hepatic regeneration reached 94% and 96% respectively (Table 2). This result and previous studies support the basis of the potential protector effect of these vitamins in their capacity to scavenge free radicals, however, the difference in percentage of hepatic regeneration suggests that vitamin E might be more efficient than vitamin C. Thus, it is important to mention that there are differences between the vitamins in their physicochemical properties. For example, vitamin C is a hydrophilic compound and a strong reductor agent which reacts with O_2 and even faster with the OH groups[43]. Therefore, the treatment with vitamin C could reduce the formation of hydroxyethyl radicals by the inhibition of oxygen radical intermediates. Besides, it has been observed *in vitro*, vitamin C is capable of reducing the 1-hydroxyethyl/PBN adducts and at the same time inhibits the adducts previously formed[44].

On the other hand, vitamin E also reacts with the oxygen radicals, but it has been observed that its efficiency depends on the radical species that is attacking. This has been shown through *in vivo* studies where it has been observed that vitamin E has more capacity to scavenge peroxyl radicals than to inhibit hydroxyl radicals[45].

Navasumrit[46] determined that a pre-treatment with vitamin E (100 mg/kg; intraperitoneal for five days) inhibits the 1-hydroxyethilic radicals generated by the ethanol, and it is more effective than pre-treatment with vitamin C (400 mg/kg; intraperitoneal for four days); attributing the result to the fact that 1-hydroxyethilic radicals are primarily generated in lipid compartments of the cell. This also supports the basis that the CYP enzymes (located in membranes of the smooth endoplasmic reticulum) are the most active site for the generation of oxygen reactive radicals, and suggest the correlation between the induction of CYP2E1 and the generation of the 1-hydroxyethilic radicals. This evidence with the fluorescent analysis (laser scanner confocal fluorescence image analysis system) of hepatocytes[47] suggests that the 1-hydroxyethilic radicals are primarily located in the hepatocyte plasma membrane and therefore the lipophilic property of vitamin E gives it a major facility to inhibit the generation of those radicals in comparison with vitamin C, which is hydrophilic (Figure 2).

On the other hand, the reduced effect of vitamin A in relation to hepatic regeneration fits with the report of Portari[42], who evaluated the action of β-carotene in the chronic administration of ethanol in rats. They conclude that because it is a pro-oxidant compound, its mechanism of action could be diminished when the serum and hepatic concentrations are low. As a consequence of this, the reduced effect observed here could be attributed to the intention of the organism to recuperate the serum concentration of this vitamin which was reduced by the extreme consumption of ethanol. To support this possibility, it is important to mention that some studies have indicated that ethanol interferes in several metabolic pathways of vitamin A, that β-carotene is a precursor of this vitamin, that retinol is the alcoholic form of vitamin A. Thus, both retinol and ethanol share the same oxidant activity, and are biotransformed by dehydrogenases, so

metabolic interactions that reduce the normal concentration of vitamin A can occur[48].

As it has already been mentioned, the chronic consumption of ethanol damages hepatic cells. The serum concentrations of albumin and bilirubin and the enzymatic activities of AST and ALT are the most sensitive tests used for the diagnosis of hepatic diseases[49]. In this study, it was observed that the rats treated with ethanol presented a decrease in the concentration of albumin, as well as an increase in the serum concentrations of bilirubin, ALT, and AST (Table 3 and Figure 1). The decrease in albumin supports the previous report, where the same dose of ethanol was applied to evaluate the hepatic regeneration and it was analyzed whether toxicity was modified when ethanol was administered intraperitoneally or intragastrically. Both studies prove the hepatotoxic potential of ethanol and show evidence that low serum albumin is directly related with a decrease in the normal ATP generated in the liver; the organ which is only affected by the ethanol and not by the partial hepatectomy where the level of this protein is comparable to the sham group[40]. On the other hand, in the groups combined with ethanol and vitamins C and E, the concentrations of serum albumin and bilirubin were almost normalized (Table 3), which confirms the protective effect of this vitamin over the free radicals generated by the ethanol, as well as its efficiency in the cellular regeneration of the liver (Table 2 and Figure 2).

The enzymatic elevation can probably be attributed to the damaged structural integrity of the hepatic cells, because these enzymes are located in the cytoplasm and are released into circulation after cellular damage. If injury involves other organelles, such as mitochondria, then the soluble enzymes such as AST compartmentalized will also be similarly released indicating that alcohol consumption causes both plasma membrane and organelle membrane damage. On the administration of vitamins to alcoholic rats we observed decreased levels of serum AST and ALT, suggesting the protection capacity of these vitamins to preserve normal structural liver (Figure 1).

On the other hand, increased serum patterns of LDH, ALT and AST activities after 70% PH in rats have been reported previously[50]. The increase of these serum enzyme activities during the onset of liver proliferation could be related to cell necrosis or enhanced cell membrane permeability. Indeed, it has been concluded that increased serum activities of some enzymes after PH could be due to early release either from damaged cells or by cells with altered permeability, probably involving enhanced synthesis and release of enzymes[40].

It is known that ethanol inhibits liver regeneration, resulting in the increase in free radicals produced by the metabolism of ethanol, causing cellular damage as well as the alteration of liver functions. Our results propose that vitamin C and E which are free radical scavengers block the inhibitory effect of regeneration caused by the ethanol by decreasing the damage in the cellular membrane (MDA). This decrease in damage caused by the free radicals produced by the metabolism of ethanol also decreased the indicators of liver damage such as TGO and TGP, as well as the ones for physiological state (albumin and bilirubin) causing an increase in the indicators of liver regeneration (restitution of the liver mass, liver/body ratio and mgDNA/g liver ratio), and consequently reducing mortality.

In conclusion, in this study, the groups treated with ethanol plus the vitamins C or E had better response in terms of liver regeneration, showing less oxidative stress, and had better liver function than the PH-EtOH and the PH-Ethanol-vitamin A groups.

COMMENTS

Background

Evidence demonstrates that oxidative stress plays an important etiologic role in the development of alcoholic liver disease. Vitamins are one of the most promising antioxidant defense systems. Recently, it has become clear that antioxidant nutrients, including vitamins, are important for the liver. In addition, evidence for alcohol-induced oxidant injury comes from studies in which pretreatment with antioxidants such as vitamin E, vitamin C, and agents that enhance antioxidant capacity attenuate alcohol induced effects.

Research frontiers

The aim of this study is to analyze the effects of vitamins A, C and E in rats with liver injury induced by ethanol administration during liver regeneration, as liver protector agents.

Innovations and breakthroughs

This study clearly shows that vitamin C and E, which are free radical scavengers, block the inhibitory effect of regeneration caused by the ethanol by decreasing the damage in the cellular membrane (MDA); causing an increase in the liver regeneration indicators (restitution of the liver mass, liver/body ratio and mgDNA/g liver ratio), and consequently reducing mortality.

Applications

The treatment of vitamin C and E can be used as a hepatoprotective agent from the damage caused by ethanol. It could be used as a protector of the damaged produced by ethanol, opening a window to new therapeutic strategies, of novel agents which correct fundamental cellular disturbances resulting from excessive alcohol consumption.

Terminology

Partial hepatectomy: the operations consisted of removal of the median and left lateral lobes of the liver. Liver regeneration after partial hepatectomy in the rat has been widely employed as an experimental model to study mammalian cell proliferation.

Peer review

This is an interesting study, aiming to identify the effects of vitamins A, C and E in rats, as hepatoprotective agents of the damage produced by ethanol during liver regeneration. The authors concluded that vitamin C and E are excellent therapeutic agents against the liver damage produced by ethanol.

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RAPID COMMUNICATION

H pylori eradication: A randomized prospective study of triple therapy with or without ecabet sodium

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Abstract

AIM: To investigate whether adding ecabet sodium to the standard triple therapy for *H pylori* infection improve eradication rate.

METHODS: Two hundred and fifty-seven *H pylori*-infected patients were randomly assigned to standard triple therapy (group A, *n* = 129) or triple therapy plus ecabet sodium (group B, *n* = 128). Successful eradication was defined as a negative ^{13}C-urea breath test 6-8 wk after completion of treatment.

RESULTS: After completion of therapy, 194/257 patients showed negative ^{13}C-urea breath test results. According to intention-to-treat analysis, the infection was eradicated in 93/129 (72.1%) patients in group A and 101/128 (78.9%) in group B (*P* = 0.204). Per-protocol analysis showed successful eradication in 93/118 (78.8%) patients from group A and 101/114 (88.6%) from group B (*P* = 0.044). There were no significant differences in the side effects experienced by the patients in the two treatment groups.

CONCLUSION: Our results suggest that the addition of ecabet sodium improves the efficacy of the standard triple therapy for *H pylori*.

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Key words: *H pylori*; Eradication; Triple therapy; Ecabet sodium

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INTRODUCTION

H pylori is a gram-negative spiral-shaped bacterium that colonizes the human stomach and it is associated with several gastroduodenal diseases, including gastritis, peptic ulcer disease and low-grade gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Eradication of *H pylori* is the recommended treatment for these conditions[1]. The most widely endorsed treatment is triple therapy with amoxicillin, clarithromycin and a proton pump inhibitor two times daily for 7 d, and the reported efficacy of this protocol is 74%-76%[2]. The reasons for its occasional failure are unclear, although bacterial resistance and poor patient compliance are believed to be the primary factors[3]. Ecabet sodium is a dehydroabietic acid derivative that was originally purified from pine resin[4], which is now widely used for the treatment of gastric ulcer and gastritis in East Asia. This agent exhibits a bactericidal effect against *H pylori* by inhibiting bacterial urease activity[5], or by its direct bactericidal effect under acidic conditions[6,7]. Its bactericidal effect on clarithromycin- and metronidazole-resistant clinical isolates of *H pylori* has been reported[7]. Therefore, ecabet sodium has been suggested to improve the efficacy of antibiotic therapy for *H pylori* infection

in patients with peptic ulcer[8-12]. To the best of our knowledge, the efficacy and safety of standard triple therapy plus ecabet sodium has not yet been investigated. Therefore, the aim of this study was to compare the efficacy and side effects of standard triple therapy versus triple therapy plus ecabet sodium for the eradication of *H pylori*.

MATERIALS AND METHODS

Patients

This randomized prospective study was performed at three medical centers (Pusan National University Hospital, Bongseng Memorial Hospital and Maryknoll Hospital, Busan, Korea). The subjects consisted of 257 consecutive *H pylori*-positive patients who had undergone eradication therapy from May 2006 to April 2007. The criteria for exclusion included: (1) previous *H pylori* eradication therapy; (2) ingestion of antibiotics, bismuth, or proton pump inhibitors within the prior 4 wk; (3) patients with a history of allergy to the medications used; (4) patients with previous gastric surgery; (5) coexistence of serious concomitant illness (for example, decompensated liver cirrhosis or uremia); and (6) pregnant women.

All the participants were advised to undergo *H pylori* eradication therapy and they all agreed. There were 148 men and 109 women (mean age 53.9 ± 12.8 years), and these included: 128 patients with gastric ulcer (or ulcer scar); 63 with duodenal ulcer (or ulcer scar); 14 with gastroduodenal ulcer (or ulcer scar); 12 who had received endoscopic treatment for early gastric cancer or adenoma; one with gastric low-grade MALT lymphoma; and 39 with gastritis accompanied by dyspepsia or a family history of gastric cancer (Table 1).

The patients were then randomized into two groups: group A (n = 129) underwent standard triple therapy for 7 d (lansoprazole 30 mg b.i.d., clarithromycin 500 mg b.i.d. and amoxicillin 1 g b.i.d.), while group B (n = 128) patients were given treatment consisting of the same triple therapy plus ecabet sodium (1.0 g b.i.d.) for a period of 7 d. Patients were instructed to take all the drugs 30 min after breakfast and dinner. This study was performed in accordance with good clinical practice and the Declaration of Helsinki guidelines. The Institutional Review Board of Pusan National University Hospital approved this study, and informed consent was obtained from all the patients.

Methods

Before receiving therapy, all the patients underwent upper gastrointestinal endoscopy to confirm the diagnosis and the presence of *H pylori* infection. Positive *H pylori* status was confirmed by the rapid urease test, histology, serology (serum IgG antibody) and/or the ^{13}C-urea breath test. The patients were diagnosed as *H pylori*-positive when they showed positive results by at least two methods.

Possible eradication of *H pylori* was assessed by the ^{13}C-urea breath test at 6-8 wk after completion of treatment. Proton pump inhibitors and antimicrobial agents that might affect the ^{13}C-urea breath test were not given to the patients after completion of therapy.

Table 1 Demographic data and endoscopic findings of 257 patients with *H pylori* infection who were randomly assigned to standard triple therapy (group A) or triple therapy plus ecabet sodium (group B)

	Group A	Group B	*P* value
Patients	129	128	
Age (yr) (mean ± SD)	54.3 ± 13.2	53.4 ± 12.4	0.562
Gender (male/female)	68/61	80/48	0.112
BMI (kg/m^2) (mean ± SD)	23.3 ± 2.9	23.2 ± 3.0	0.768
Smoking	32 (24.8%)	32 (25.0%)	0.971
Alcohol consumption	40 (31.0%)	56 (43.8%)	0.035
Endoscopic findings			0.333
Gastric ulcer	62	66	
Duodenal ulcer	37	26	
Gastroduodenal ulcer	8	6	
Gastric cancer[1]	7	5	
Gastric MALT lymphoma	0	1	
Gastritis	15	24	

[1]Patients who had received endoscopic treatment for early gastric cancer or adenoma.

The ^{13}C-urea breath test was performed as described previously, with capsule-based modification[13]. In brief, the patients fasted for over 12 h before examination and then a gelatin capsule containing 38 mg ^{13}C-urea was ingested with 50 mL water. Breath samples before and 20 min after administration of ^{13}C-urea were collected after a mouthwash. The ^{13}C/^{12}C ratio in the breath samples was measured by mass spectrometry (Heliview; MediChems, Seoul, Korea). The changes in the ^{13}C value over baseline were expressed as Δ^{13}C. A positive result was defined as an increase of > 2‰.

The eradication rate was evaluated for each drug regimen in a per-protocol (PP) analysis based on the number of patients who completed the study, and in an intention-to-treat (ITT) analysis that took into account all the patients, including those who dropped out because of severe side effects, those who showed poor drug compliance, and those who were lost to follow-up. The patients were interviewed in their first visit to the clinic after completion of therapy to clarify their compliance with the therapy and any side effects. Poor compliance was defined as taking less than 70% of the total medication.

Statistical analysis

χ^2 or Fisher's exact tests were used to compare the characteristics of the patients and the results of two regimens. Independent t tests were also employed to compare the possible differences in the age and body mass index of the patients who were treated with the two regimens. $P < 0.05$ was considered statistically significant. Statistical calculations were performed using SPSS version 12.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Group A consisted of 129 patients (68 men and 61 women, mean age 54.3 ± 13.2 years) and group B consisted of 128 patients (80 men and 48 women, mean age 53.4 ± 12.4 years). Data regarding the clinical

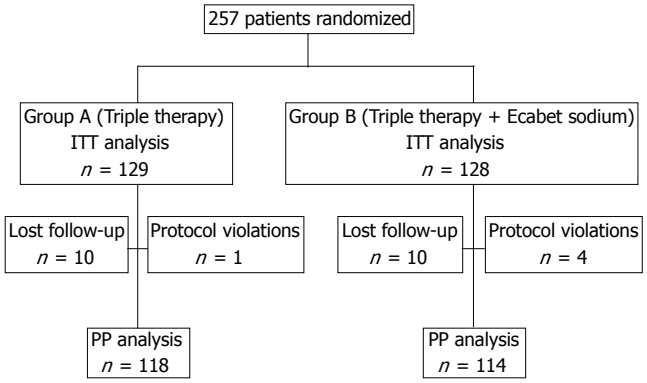


Figure 1 CONSORT flow diaphragm of the subjects' progress through the phases of the study.

characteristics of the patients at entry are summarized in Table 1. The two groups had a comparable age, gender and history of smoking. Alcohol consumption was more common in group B than in group A. There were no significant differences in the endoscopic findings between the two groups.

Five patients dropped out due to non-compliance before the final evaluation: three patients due to side effects (one from group A and two from group B) and two patients due to personal reasons. A further 10 patients from each group did not appear at the first visit after completion of therapy or for the ^{13}C-urea breath test; therefore, they were lost to follow-up (Figure 1).

After the completion of therapy, 194/257 (75.5%) patients tested negative for *H pylori* on the ^{13}C-urea breath test. ITT analysis showed that *H pylori* was eradicated in 93/129 (72.1%) patients from group A and in 101/128 (78.9%) patients from group B (P = 0.204). PP analysis showed successful eradication in 93/118 (78.8%) patients from group A and 101/114 (88.6%) patients from group B (Table 2, P = 0.044).

There were no significant differences in the side effects experienced by the patients in the two treatment groups: 26/119 (21.8%) patients in group A and 24/118 (20.3%) patients in group B reported adverse effects due to therapy (Table 3, P = 0.776). More patients in group A complained of a metallic taste than in group B (11.8% *vs* 4.2%, P = 0.033). All the side effects were self-limiting and disappeared once therapy was terminated.

Table 2 ITT and PP analysis of *H pylori* eradication in patients who were randomly assigned to standard triple therapy (group A) or triple therapy plus ecabet sodium (group B)

	Group A	Group B	*P* value
Eradication rate			
ITT[1]	72.1% (93/129)	78.9% (101/128)	0.204
PP	78.8% (93/118)	88.6% (101/114)	0.044

[1]In this analysis, the patients with unknown outcome were counted as treatment failures.

Table 3 Side effects during standard triple therapy (group A) and triple therapy plus ecabet sodium (group B)

Side effects	Group A	Group B	*P* value
Metallic taste	14	5	0.033
Nausea/vomiting	1	4	0.213
Abdominal pain	2	2	1.000
Constipation	1	0	1.000
Diarrhea	7	3	0.333
Dizziness	1	6	0.066
Skin rash	0	3	0.122
Oral mucositis	2	1	1.000
Others	4	7	0.347

DISCUSSION

Reports about failed *H pylori* eradication therapy have been increasing[14,15]. A recent meta-analysis has demonstrated that a 1-wk course of triple therapy with amoxicillin, clarithromycin and a proton pump inhibitor had a pooled eradication rate of 74%-76% or less[2,16]. A disappointing cure rate of < 80% after 7 d triple therapy was confirmed in the present study.

Various studies are under way to evaluate new antibiotics (e.g., levofloxacin, moxifloxacin, furazolidone and rifabutin) and different therapeutic schedules to increase the efficacy of *H pylori* eradication therapy[17-20]. Data suggest that antibiotic resistance is frequent and clinicians are arriving at the conclusion that concurrent therapy with another agent may sometimes be necessary.

Ecabet sodium has local cytoprotective activity for the gastric mucosa, but is not absorbed into the systemic circulation[21]. Recent studies have demonstrated that ecabet sodium has anti-*H pylori* activity. *In vitro* studies have shown that ecabet sodium inhibits the urease activity of *H pylori* and its adhesion to the gastric epithelial cells[5,22]. In addition, ecabet sodium is reported to have a strong concentration-dependent bactericidal effect on *H. pylori* under acidic conditions[7]. Although proton pump inhibitors potently suppress acid secretion, the acid milieu (pH 3-5) is especially maintained during most of the day and night[23]. Therefore, several studies have evaluated the usefulness of ecabet sodium for eradication therapy in combination with proton-pump-inhibitor-based therapy[8,10,12].

A comparative study demonstrated an eradication rate of 26% with lansoprazole-based dual therapy with clarithromycin or amoxicillin, whereas the eradication rate achieved by adding ecabet sodium to the same regimen was 79%[12]. Another study confirmed the additive effect of ecabet sodium on proton-pump-inhibitor-based dual therapy[11]. In addition, a dose-dependent additive effect of ecabet sodium in combination with lansoprazole and amoxicillin has been demonstrated[10].

On the basis of this evidence, we thought it likely that the addition of ecabet sodium to triple therapy contributes to improving the rate of *H pylori* eradication. In fact, in this study, PP analysis showed that triple therapy plus ecabet sodium was more effective than standard triple therapy, although there was no significant difference according to ITT analysis. It is possible that triple therapy plus ecabet sodium provokes a synergistic effect by launching attacks from different directions against *H pylori*, and this may

lead to complete clearance of the bacterial infection. The bactericidal effect of ecabet sodium on clarithromycin- and metronidazole-resistant clinical isolates of *H pylori*[7] might also explain the increased eradication rate.

The majority of the recently recommended eradication therapy regimens are 1 wk in duration because these have advantages with respect to compliance and medical cost, with a similar eradication rate[24]. A good eradication rate was observed in this study after a 1-wk-long triple therapy plus ecabet sodium.

Because ecabet sodium does not have any systemic activity, it was expected that ecabet sodium-based therapy would be associated with a low occurrence of adverse events. In a previous report, the use of ecabet sodium combined with lansoprazole and amoxicillin reduced the incidence of diarrhea during therapy[11]. However, there was no significant difference of adverse events between standard triple therapy and triple therapy plus ecabet sodium in this study. The use of triple therapy plus ecabet sodium reduced the incidence of metallic taste during therapy, and this might have been due to the sweet taste of ecabet sodium.

In recent years, many studies have compared the effectiveness of triple and bismuth quadruple therapy as a primary therapy for *H pylori*[25-28]. A recent meta-analysis including five randomized trials has reported slightly higher, but not statistically significant, eradication rates with quadruple therapy: ITT and PP eradication rates were 79% and 85% for clarithromycin triple therapy and 80% and 87% for bismuth quadruple therapy, respectively[29]. However, quadruple therapy is complex to take (q.i.d. dosing regimen and high pill count) and has a perceived high frequency of side effects[30].

In conclusion, this randomized prospective study showed (based on PP analysis) that the addition of ecabet sodium improved the efficacy of standard triple therapy for *H pylori* infection. These results need to be confirmed, but triple therapy plus ecabet sodium may represent a valid alternative regimen to the standard *H pylori* eradication protocol.

COMMENTS

Background

The eradication rate of *H pylori* by standard triple therapy is only 74%-76%. Ecabet sodium has been reported to exhibit a bactericidal effect against *H pylori* by inhibiting bacterial urease activity or by its direct bactericidal effect. This study compared the efficacy and side effects of standard triple therapy versus triple therapy plus ecabet sodium for the eradication of *H pylori*.

Research frontiers

Research in this area is focused on developing more effective eradication regimens for *H. pylori* infection. Ecabet sodium is widely used for the treatment of gastric ulcer and gastritis in East Asia. Its bactericidal effect on clarithromycin- and metronidazole-resistant clinical isolates of *H pylori* has been reported. Therefore, it is presumed that adding ecabet sodium to standard triple therapy is more efficient for the eradication of *H pylori*. In our randomized prospective study, the addition of ecabet sodium may have improved the efficacy of standard triple therapy for *H pylori*.

Innovations and breakthroughs

Some reports about the efficacy of ecabet sodium for *H pylori* infection have been reported. But the efficacy and side effects of standard triple therapy plus ecabet sodium have not yet been investigated. By PP analysis, the rate of successful eradication was 78.8% for standard triple therapy and 88.6% for triple therapy plus ecabet sodium. There were no differences in the side effects in the two treatment groups. These results need to be confirmed, but triple therapy plus ecabet sodium may improve the eradication rate of *H pylori*.

Applications

This study demonstrated the efficacy of standard triple therapy plus ecabet sodium. Therefore, this may represent a valid alternative regimen to the standard *H pylori* eradication protocol.

Terminology

Ecabet sodium is a dehydroabietic acid derivative purified from pine resin and one of the local cytoprotective agents for the gastric mucosa. It is now widely used for the treatment of gastric ulcer and gastritis in East Asia. The eradication rate was evaluated for each drug regimen by PP analysis, based on the number of patients who completed the study, and in an ITT analysis that took into account all patients.

Peer review

This study compared two therapeutic regimens for the cure of *H pylori* infection: a classic triple PPI based therapy *vs* the same regimen plus ecabet sodium (a natural bactericidal compound). The study was well designed and conducted.

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RAPID COMMUNICATION

Non-alcoholic fatty liver disease in the Philippines: Comparable with other nations?

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Abstract

AIM: To evaluate the prevalence and determined the common characteristics of patients diagnosed with non-alcoholic fatty liver disease (NAFLD) at the Philippine General Hospital, Manila, from January 1999 to December 2004.

METHODS: NAFLD was diagnosed in 134 from a total of 1102 patients, based on clinical, ultrasonographic and/or histopathological findings. Patients with conditions associated with secondary NAFLD were excluded. Chart review was done to note demographics, co-morbid illnesses, physical characteristics, hepatomegaly, aspartate/alanine aminotransferase (AST/ALT) levels, albumin, lipid levels, alkaline phosphatase, prothrombin time, and partial thromboplastin time. Data obtained were analyzed using the statistical program SPSS version 10.

RESULTS: Of the 134 patients included, 71% were female and 29% male. Mean patient age was 42.2 years. Sixty percent of patients were obese, 56% had hepatomegaly, and 69% had diabetes. AST levels were elevated in 45% of subjects and ALT levels in 64%.

CONCLUSION: The prevalence of NAFLD at our institution was 12.2%. Patients diagnosed appear to be younger in age in contrast to previous studies. Female sex, obesity, elevated liver enzymes, and diabetes were characteristic features of our NAFLD patients, which is comparable to previous studies from other countries.

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Key words: Fatty liver; Obesity; Non-alcoholic fatty liver disease; Diabetes; Philippines

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is now recognized as one of, if not the most common liver diseases in the United States, with a prevalence of about 5% in the general population, and reaching 25%-75% in patients with obesity and type 2 diabetes mellitus. Data regarding prevalence in the general population of the Philippines have been lacking. NAFLD covers a spectrum of diseases that ranges from simple fatty deposition in the liver to inflammation, fibrosis and cirrhosis. Although the exact etiology is not clear, it may be a part of a metabolic syndrome associated with insulin resistance, diabetes, obesity and hypertension. Patients typically present with asymptomatic serum aminotransferase elevation of 2-3 times the normal level. Symptoms may include fatigue and abdominal pain. Physical examination may show hepatomegaly. Liver biopsy is useful and effective as a prognostic indicator, but it is an invasive and costly tool to diagnose fatty liver, and is mainly used for unexplained cirrhosis. NAFLD can be diagnosed by histology, ultrasonography, CT, and MRI. However, the imaging modalities most often used to identify hepatic steatosis include CT and ultrasonography. Hyperechogenic (bright) liver indicates steatosis, with sensitivity and specificity rates as high as 95%. The prevalence of obesity has been steadily rising in the Philippines, which may be attributed to a change in dietary habits and a sedentary life style.

NAFLD includes a spectrum of liver diseases, including fatty liver, steatohepatitis, and even cirrhosis in the absence of alcohol intake, that are known to be injurious to the liver. It has a benign course in the majority of cases, but up to 20% of these patients with NAFLD have non-alcoholic steatohepatitis NASH[1,4]. Up to one-third of these NASH cases can progress to cirrhosis, liver failure and even hepatocellular carcinoma[2]. It is now believed to be a widespread liver disease in Western countries, and most probably, even in Asian countries. Moreover, it may be the leading cause of cryptogenic cirrhosis[3,5]. Even with its preponderance, NAFLD remains something of a medical mystery. Recent studies have shown an upsurge of

interest in this liver disease and it is becoming increasingly significant[6].

A lot of work has been done on NAFLD in Western countries[6-8], but it is relatively new in the Philippines, with only a handful of studies having been done. Recent studies have proposed that obesity and type Ⅱ diabetes mellitus, which are hyperinsulinemic states with decreased tissue sensitivity to insulin, are the major players in the pathogenesis of NAFLD. This study was conducted to note characteristic features in our NAFLD patients at the Philippine General Hospital, Manila, confirmed by clinical, ultrasonographic and/or histopathological findings, and to establish if these findings were congruent with those of previous studies on NAFLD.

MATERIALS AND METHODS

This study was conducted at the Philippine General Hospital, Manila, Philippines based on case records from January 1999 to December 2004. From a total of 1102 patients with fatty liver, 134 patients were diagnosed with NAFLD, based on clinical and histopathological findings or ultrasound findings suggestive of fatty liver. Fatty liver was diagnosed by ultrasonography using an abdominal probe at 2-5 MHz. Longitudinal, subcostal, ascending, and oblique scans were performed. The ultrasonographic criteria that were used to diagnose fatty liver included liver and kidney echo discrepancy, presence of increased liver echogenicity (bright), echo penetration into the deep portion of the liver, and clarity of liver blood vessel structures. Due to the subjectivity and vagueness of some of the case records reviewed on the amount of alcohol consumption, patients included had absolutely no history of alcohol intake. HCV-antibody-positive patients were excluded. Patients with a history of various surgical procedures that can lead to secondary NAFLD, i.e. gastropexy, jejunoileal bypass, extensive small bowel resection, biliopancreatic diversion, and small bowel diverticulosis, were excluded, as were patients taking drugs or conditions that are known to cause secondary NAFLD.

Chart review was performed to note history, demographics, co-morbid illnesses, physical characteristics, physical examination findings, and laboratory findings. Detailed clinical evaluation of each subject was carried out to note patient characteristics that focused on primary causes of NAFLD. Presence of obesity, hepatomegaly, and diabetes were specifically sought. Body mass index (BMI) of each subject was noted to establish obesity. Patients with BMI < 18, 18-25, 26-29 and ≥ 30 were considered under-weight, normal, over-weight and obese, respectively. Fasting serum cholesterol and triglyceride levels of all patients were noted for lipid abnormalities, but a substantial lack of data precluded their inclusion in this study. Fasting blood sugar level in all patients was noted. Diagnosis of diabetes in these patients was made according to standard criteria.

In most patients' medical charts, serum level of alanine/aspartate transaminases (ALT/AST), albumin, prothrombin time, partial thromboplastin time, and alkaline phosphatase were documented. Data from study participants obtained from medical records included age, gender, nationality, weight, height, BMI, and clinical presentation or presence of hepatomegaly. The results were expressed as means ± SD. Data obtained was then converted into variables that were analyzed using frequency and cross tabulation functions of computer-based statistical program SPSS version 10. The test was considered to be significant if P was < 0.05.

Table 1 Distribution of BMI *n* (%)

Gender	Underweight	Normal	Overweight	Obese
Female	10 (11%)	5 (6%)	8 (9%)	64 (74%)
Male	17 (36%)	10 (21%)	4 (9%)	16 (34%)
Total	27 (20%)	15 (11%)	12 (9%)	80 (60%)

RESULTS

Demographics and physical attributes

Of the 1102 patients diagnosed with fatty liver, 134 (12.2%) met the criteria for review. As expected, a huge proportion of the cases came from metropolitan Manila (69%), with the rest of the cases coming from the southern Luzon regions (21%). Results showed that 71% of the subjects were female and 29% were male. Mean patient age was 42.2 ± 2.1 years. Half of the patients were aged 21-47 years. Mean BMI was 31.8 ± 7.2, with 60% of patients being obese or having BMI > 30, while 11% had normal BMI, 9% were overweight and 20% were underweight. Most obese patients (80%) were female (Table 1).

Clinical features

Fatigue and right upper quadrant discomfort were the most common symptoms seen in both male (34%) and female (29%) patients. Most however, had no symptoms at all (62%). Overall, a greater proportion of subjects with normal ALT were completely asymptomatic compared with those with abnormal ALT (P < 0.04, Fisher's exact test). Approximately 59% of subjects had a relatively unremarkable physical examination, with the exception of their body weight and blood pressure. A large number of NAFLD patients (92; 69%), had diabetes. Hypertensive patients accounted for 22% (29) of the sample population. Twenty-seven patients with NAFLD and normal ALT were obese, as defined by BMI > 30. Twenty-nine patients with NAFLD and normal ALT levels had diabetes, and 14 had hypertension. One patient with normal and one with elevated ALT had hypothyroidism. None of the subjects were taking medication known to be associated with the development of hepatic steatosis.

Laboratory correlation

Based on autopsy, ultrasound or physical examination, 75 (56%) patients had hepatomegaly. A total of seven autopsied patients who met the criteria for NAFLD had an average liver weight of 1722 ± 488 g (normal 1500-1800 g). AST levels were elevated in 60 (45%) of subjects and ALT levels in 86 (64%). AST levels averaged 112 ± 20 U/L for females and 164 ± 27 U/L for males. ALT levels averaged 217 ± 18 U/L for females and 187

Table 2 Laboratory characteristics of NAFLD patients at the Philippine General Hospital

Parameter	Blood chemistry values		
	Total *n* = 134 (mean)	Male *n* = 39 (mean)	Female *n* = 95 (mean)
Liver weight (g)	1722 ± 488	1505 ± 399	1952 ± 512
AST (U/L) *n* (15-137)	150 ± 19	164 ± 27	112 ± 20
ALT (U/L) *n* (30-65)	192 ± 17	187 ± 24	217 ± 18
Alkaline phosphatase (g/dL) *n* (50-136)	89 ± 20	87 ± 30	92 ± 22
Albumin (g/L) *n* (34-50)	33.5 ± 6	35 ± 8	32 ± 6
Prothrombin time (PT) INR	1.1 ± 1	0.95 ± 1	1.2 ± 1
	Median (range)	Median (range)	Median (range)
PT activity %	88 (79-99)	90 (79-99)	88 (90-97)
Partial thromboplastin time (s)	30.1 (28-49.1)	32 (28-40.3)	34.6 (33-49.1)
Random blood sugar mmol/L *n* (3.9-6.1)	8.3 (4.9-20.1)	7.6 (5.5-20.1)	8.3(4.9-18.2)

Table 3 Comparison between studies of the characteristic features of NAFLD patients

Study	No. of patients	Age (yr)	Female (%)	Diabetes (%)	Obesity (%)	Hepatomegaly (%)
Ludwig (1988)[18]	20	54	65	50	90	67
Diehl (1988)[28]	39	52	81	55	71	20
Lee (1989)[25]	49	53	78	51	69	-
Powell (1990)[26]	42	49	83	36	95	81
Bacon (1994)[27]	33	47	42	21	39	21
Matteoni (1999)[29]	132	53	53	33	70	92
Khurram (2003)[31]	50	47	54	44	66	76
Our study (2005)	134	42	71	69	60	56

± 24 U/L for males. Bleeding abnormalities were noted in 26 (19%) patients. Mean blood glucose level for male patients was 6.7 ± 8 mmol/L and 8.1 ± 5 mmol/L for females. Alkaline phosphatase and albumin levels were within the normal range for the whole sample population. Glutamyltransferase levels and lipid profiles were not included due to scarcity of available data from charts reviewed. Details are shown in Table 2.

Risk factors

Female sex, diabetes, hepatomegaly, increased BMI (overweight and obesity) were the main risk factors noted to have a significant relationship with NAFLD ($P < 0.05$). Significant differences ($P < 0.05$) in BMI, liver weight, AST, ALT and blood sugar were also noted between female and male individuals. It was notable that a sizable proportion of our patients (20%) who were underweight also had had NAFLD. No significant relationship was found between the presence of fatty liver and age. A significant number (92; 69%) of our patients had diabetes, 69 (75%) of whom were female. The mean duration of diabetes was 9.9 ± 3.9 years. Mean HbA1C was elevated at 9.1% ± 2.6%. Hepatomegaly with fatty infiltration, as assessed by ultrasound, was reported in 66 patients (49%) with a mean liver size of 17.4 ± 5.2 cm.

DISCUSSION

NAFLD includes a wide spectrum of histological abnormalities that are similar to alcoholic liver disease, but without a prior history of alcoholic intake known to be injurious to the liver. NAFLD is a common problem in the US, Asia-Pacific region, Europe, and the Middle East. NAFLD can affect any age group but is usually encountered in the 40-60 years age group[5]. Prevalence is high even among children, with 2.6% of all children and up to 52.8% of obese children having this disease[3]. Several investigators have noted that the mean age of patients with NAFLD was 52-54 years[18,25,28,29]. In other studies mean age varied between 47 and 54 years of age[26,27,31]. Most of our patients were aged 21-47 years, which is considered a younger age group. This may reflect the changing diet of young Filipinos to caloric rich foods, which has increased the number of people with diabetes in the Philippines, or it may just reflect the limitation of our sample population.

Most of our other results, including the presence of diabetes, hepatomegaly and obesity, correlated with the findings of previous authors[18,26-30]. Up to 65%-80% of females may have this disease, as shown in our study and in some previous studies, with the exception of that of Bacon *et al*, in which there were only 42% females[18,26-30]. In our study, females constituted 71% of patients. Different studies have shown a gender-related difference in NAFLD-associated conditions such as hepatomegaly, diabetes and dyslipidemia; these conditions being more common in female NAFLD patients. Our findings were similar in most cases, i.e. 64% patients with obesity were female and 69 females or 51% of the whole population had diabetes. Diabetes mellitus is an independent risk factor for liver-related deaths in NAFLD patients[3]. In related studies, up to 75% of NAFLD patients had diabetes[18,26-30]. Our results agreed with these studies, with 60% of our population having diabetes, and there was a significant correlation with NAFLD ($P < 0.05$). It has been noted by Angulo that the prevalence of NAFLD increases by a fraction of 4.6 in patients with obesity, with up to 74%

of such patients having NAFLD[3]. In previous studies, 30% to 81% of patients diagnosed with NAFLD were found to have obesity[18,26-30]. Our results correspond with these studies, with a mean BMI of 31.8 ± 7.2. This was also significantly correlated with the presence of NAFLD ($P < 0.05$). Several studies have shown that truncal obesity is an important risk factor for development of NAFLD, even in patients with normal BMI. Truncal obesity ideally should have been included in the data collection but was not incorporated due to lack of data in case records.

On the other hand, fatty liver is associated with mild to moderate enlargement of the liver. Hepatomegaly is commonly the sole physical finding in most patients with NAFLD, which was also seen in our patients. Similar results have been found in other related studies[18,26-30]. Hepatomegaly may even be more severe due to the fact that normal values used for comparison come mainly from Caucasian data, and may not reflect true normal values in Filipinos. Hyperlipidemia has also been tagged from previous studies as a risk factor for the development of NAFLD[18,26-30]. Prevalence of hyperlipidemia in NAFLD patients is quite variable. Diehl *et al* noted it in only 20% of NAFLD patients, contrary to the results of Matteoni et al who found it in 92% of patients[28,29]. Unfortunately, our study was not able to document lipid profiles due to the non-availability of laboratory results. Characteristic features of our NAFLD patients compared with other studies are given in Table 3.

Limitations include the location of our study, which was a large tertiary hospital in the Philippines. This may not be a representative sample population for analysis. Moreover, not all cases of NAFLD were analyzed, because of the lack of data from case records. Results may have differed with inclusion of these patients. Furthermore, normal values used for BMI and hepatomegaly are based mostly on Caucasian data and may not be applicable to the Filipino population. Larger prospective studies with control groups for longer periods of time are needed to firmly establish the validity of our results.

In conclusion, NAFLD was common in our patients, with a mean age of 42 years. In a 5-year period, more females (71%) were diagnosed with NAFLD. Hepatomegaly is the most common physical finding and was noted in 56% of our patients. A younger mean age, obesity (60%), diabetes (69%), hepatomegaly (56%) and female sex (71%) were the most distinct attributes of our patients with NAFLD. Except for a younger age group, the results are comparable to previous studies from other countries.

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RAPID COMMUNICATION

Melatonin reduces bacterial translocation and apoptosis in trinitrobenzene sulphonic acid-induced colitis of rats

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Abstract

AIM: To investigate the effects of exogenous melatonin on bacterial translocation and apoptosis in a rat ulcerative colitis model.

METHODS: Rats were randomly assigned to three groups: group Ⅰ: control, group Ⅱ: experimental colitis, group Ⅲ: colitis plus melatonin treatment. On d 11 after colitis, plasma tumor necrosis factor-α, portal blood endotoxin levels, colon tissue myeloperoxidase and caspase-3 activity were measured. Bacterial translocation was quantified by blood, lymph node, liver and spleen culture.

RESULTS: We observed a significantly reduced incidence of bacterial translocation to the liver, spleen, mesenteric lymph nodes, portal and systemic blood in animals treated with melatonin. Treatment with melatonin significantly decreased the caspase-3 activity in colonic tissues compared to that in trinitrobenzene sulphonic acid- treated rats (16.11 ± 2.46 *vs* 32.97 ± 3.91, $P < 0.01$).

CONCLUSION: Melatonin has a protective effect on bacterial translocation and apoptosis.

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Key words: Melatonin; Colitis; Bacterial translocation; Apoptosis

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INTRODUCTION

Inflammatory bowel diseases (IBDs) are chronic relapsing conditions of unknown etiology with a high morbidity and remain largely incurable. Crohn's disease (CD) and ulcerative colitis (UC) are collectively termed as IBDs, which are characterized by chronic inflammation in the mucosal membrane at various sites in the gastrointestinal tract with periods of remission and relapse over many years. IBDs are characterized by tissue edema, increased gut epithelial permeability and excessive infiltration of the gut by inflammatory cells from the circulation. This influx results in further tissue-destructive inflammatory processes[1].

The gastrointestinal epithelium forms a barrier that separates the finely regulated homeostasis of the body interstitium from the harsh environment of the intestinal lumen[2]. In IBDs, the intestinal mocosal barrier is disrupted by inflammation and ulceration. In these circumstances, translocation of enteric bacteria and their products through the bowel wall to extra-intestinal sterile sites may occur[3]. Bacterial translocation may cause secondary infection of intra-abdominal inflammatory processes, such as intra-abdominal abscesses, or peritonitis. With regard to endotoxemia and its relationship with severity of disease, increased intestinal epithelial permeability precedes clinical relapse[4], showing that a permeability alteration may be an early event in disease reactivation[2].

The exact pathogenesis of IBD is poorly understood, but there is evidence that IBD involves interactions between immune system, genetic susceptibility and the environment[1]. Recent studies have shown the important role of antiinflammatory and antioxidant agents, including melatonin, in IBDs[5,6]. Melatonin (5-methoxy-N-acetyltryptamine), a derivative of the essential amino acid tryptop-

han, is an indolamine hormone with its blood levels varied throughout the day[7]. Melatonin is an agent that promotes sleep and is produced at night by the pineal gland. While produced primarly in the pineal gland, melatonin can also be found in cells of the bone marrow and gastrointestinal tract and plays a fundamental role in the neuroimmunoendocrine system[8]. In most of the published studies, an antioxidative effect, improved microcirculation and a stimulation of intestinal epithelium may also apply in the beneficial effects of melatonin on the symptoms of colitis induced in rats[5,9]. The purpose of this study was to determine whether exogenously administered melatonin has any antioxidative and antiinflammatory effects on the impairment of bacterial translocation and apoptosis in an experimental colitis model of rat. To our knowledge, our study is the first one showing the relation between colitis, melatonin, and bacterial translocation.

MATERIALS AND METHODS

Animals

Male Wistar-albino rats (Erciyes University Hakan Cetinsaya Experimental and Clinical Research Center, Kayseri, Turkey) weighing 225-290 g were fed *ad libitum* with a nutritionally balanced rodent diet and water. The rats were kept under constant environmental conditions. Only water was provided during the 12 h preceding the experiments. The animals used in this study were handled and treated in accordance with the strict Guiding Principles of the National Institution of Health for Experimental Care and Use of Animals. The Animal Care Committee of the University of Erciyes approved all experimental procedures in this study.

Experimental design

Thirty Wistar-albino rats (28-32 wk old) were used in the study. The rats were divided into 3 groups (10 in each group): group I (control) = normal animals, group Ⅱ (colitis) = induction of experimental colitis without further treatment, group Ⅲ (colitis + melatonin) = induction of experimental colitis plus administration of melatonin.

Experimental procedures

Colitis was induced as previously described[10]. Briefly, the rats were lightly anesthetized with halothane, and an infant feeding tube (Unoplast A/S DK 330; Hundested, Denmark) fitted onto a blunt 18-gauge needle was inserted rectally. The tip of the tube was placed nearly 8 cm into the colon and 25 mg 2, 4, 6-trinitrobenzene sulphonic acid (Sigma, St. Louis, Missouri) mixed with 0.25 mL 30% ethanol (TNBS-E) was intracolonically instilled. After instillation, the rats were supported at the supine position until recovery from anesthesia to prevent immediate anal leakage of the instillate. The rats in the control group received intracolonic saline. During the 10 subsequent days, the animals in group Ⅲ were treated with melatonin (10 mg/kg per day) intraperitoneally. Rats in the control and colitis groups received the same volume of steril saline at the same time points. All injections were given after topical application of an antiseptic solution of 10% povidone iodine.

All rats were fed with standard rat chow for 10 d. On the 11th d, all animals underwent operation. Rats were anesthetized by intraperitoneal injection of ketamine-HCl (10 mg/kg; Ketalar®, Eczacibasi, Istanbul, Turkey) and xylazine (3 mg/kg; Rompun® 2%, Bayer, Germany). The abdominal skin was cleansed with povidone-iodine after shaving. All procedures were performed under sterile conditions by a surgeon. The abdomen was opened with a midline incision, 2-mL portal and 2-mL systemic blood samples were collected for biochemical analyses and blood cultures. Samples of liver, spleen, and mesenteric lymph nodes (MLNs) were taken for the determination of bacterial translocation. Colon tissue was sampled for analysis of myeloperoxidase (MPO) and caspase-3 activity as well as for histologic examination. At the end of surgical procedure, all rats were sacrified by overdose ether inhalation.

Determination of serum tumor necrosis factor-α level

The levels of serum tumor necrosis factor-α (TNF-α) was measured using a specific enzyme-linked immunosorbent assay kit (The BioSource Rat TNF-α ELISA). The results were expressed as pg/mL.

Assay of plasma endotoxin level

Plasma endotoxin level in portal venous blood was assayed using quantitative modification of the Limulus-amebocyte-lysate test (Sigma)[11]. The results were expressed as pg/mL.

Bacterial translocation determination

Portions of the liver, spleen and MLNs were immediately homogenized in saline. Aerobic cultures were performed with blood and eosine-methylene blue agar at 37℃ for 24 h. Anaerobic cultures were performed using anaerobic blood agar in Gas-Pak jars at 37℃ for 24 h. Colonies were identified with standard microbiologic methods. Blood samples (1 mL) were cultured in tryptic soy medium at 37℃ for 7 d. Positive cultures were transferred to blood and EMB agar. Colonies were identified with standard bacteriologic methods.

Assesment of colonic damage

The distal colon was removed and opened longitudinally. Fecal material was rinsed with a gentle spray of 0.9% saline. The freshly opened colonic segments were pinned out onto a wax block and examined by a pathologist who was blinded to the treatment.

Colon macroscopic score

The extent of mucosal damage was assessed using the colon macroscopic scoring system of Wallace *et al*[12]. Macroscopic inflammation scores were assigned based on clinical features of the colon using an arbitrary scale ranging from 0-10 as follows: 0: normal appearance, 1: hyperemia, 2: hyperemia and thickening without ulceration, 3: ulceration at a single site, 4: two or more sites of ulceration or inflammation, 5: ulceration or inflammation extending > 1 cm along the colon, 6-10: damage covering > 2 cm along the colon, with the score being increased by 1 for each additional centimeter of involvement.

Colon microscopic score

The tissue samples were fixed with 10% formaldehyde solution for 24 h and transferred to 70% ethanol solution. The tissue samples were embedded in paraffin and then cut into 4-μm thick sections. Histologic examination was performed under light microscope after hematoxylin and eosin staining. A pathologist who was unaware of the animals'groupings evaluated the specimens. Tissue samples were assessed for the presence and activity of colitis as well as the extent of tissue damage using a large number of serial sections. Colonic inflammation was assessed by using a modification of the histopathologic grading system of Macpherson and Pfeifer[13]. The following criteria were used for scoring colonic damage: grade 0: no damage; grade 1: mild mocosal and/or submucosal inflammatory infiltrate and edema: punctuate mucosal erosions often associated with capillary proliferation, muscularis mucosa intact; grade 2: grade 1 changes involving 50% of the specimen; grade 3: prominent inflammatory infiltrate and edema frequently with deeper areas of ulceration extending through the muscularis mucosa into the submucosa, rare inflammatory cells invading the muscularis propria but without muscle necrosis; grade 4: grade 3 changes involving 50% of the specimen; grade 5: extensive ulceration with coagulative necrosis bordered inferiorly by numerous neutrophils and lesser numbers of mononuclear cells, necrosis extending deeply into the muscularis propria; grade 6: grade 5 changes involving 50% of the specimen.

Myeloperoxidase measurement in colon tissue

After being scored, a sample of distal colon was frozen for subsequent measurement of MPO activity as an index of granulocyte infiltration[12]. The method described by Bradley *et al*[14] was used to measure the MPO activity in colon homogenates. The previously frozen colon tissue samples were homogenized for 30 s in 4 mL of 20 mmol/L potassium phosphate buffer (pH 7.4), and centrifuged at 40 000 × *g* for 30 min at 4℃. The pellet was resuspended in 4 mL of 50-mmol/L potassium phosphate buffer (pH 6.0), containing 0.5 g/dL hexadecyltrimethyl ammonium bromide. Samples were sonicated for 90 s at full power, incubated in a 60℃ water bath for 2 h and centrifuged. The supernatant (0.1 mL) was added to 2.9 mL of 50-mmol/L potassium phosphate buffer (pH 6.0), containing 0.167 mg/mL dianisidine and 0.0005% hydrogen peroxide. Absorbance of 460 nm of visible light (A_{460}) was measured for 3 min. The results were expressed as units of enzyme activity per gram of wet tissue weight (U/g tissue).

Caspase-3 proteolytic activity

The caspase-3 activity ratio was calculated by colorimetric assay (Chemicon International, Temecula, CA). Cells harvested from different treatment groups were lysed and 200 μg protein was tested for protease activity by adding a caspase-specific substrate peptide, DVED-pnitroaniline. Caspase-3 cleavage of the peptide released the chromophore p-nitroaniline (p-NA), which was quantitated spectrophotometrically at 405 nm. The level of caspase-3 enzymatic activity in the cell lysate was directly proportional to the color reaction. The results are expressed as the fold increase of caspase activity in apoptotic cells relative to their respective controls. In background reactions, no DVED-p-NA substrate was added, and the values obtained were subtracted from experimental results before the fold increase was calculated [15].

Table 1 Comparison of macroscopic and microscopic colonic damage scores (mean ± SD)

Colonic damage score	Control	Colitis	Colitis + melatonin	*P*
Macroscopic score	0.40 ± 0.38[b]	7.1 ± 1.34	2.4 ± 0.67	< 0.01
Microscopic score	0.6 ± 0.79[a]	4.9 ± 1.23	2.0 ± 0.66	< 0.05

[b]$P < 0.01$ *vs* colitis and colitis + melatonin groups; [a]$P < 0.05$ *vs* colitis and colitis + melatonin groups.

Statistical analysis

The data are presented as mean ± SD. One-way analysis of variance (ANOVA) was used to compare serum TNF-α, endotoxin, and colon MPO and caspase-3 levels among the 3 groups. The Tamhane test was used as the post hoc test for multiple comparisons between groups. Non-parametric Kruskal-Wallis test was used to compare bacterial translocation, and macroscopic and microscopic damage scores. The data were analyzed with the Statistical Package for the Social Sciences (SPSS) for Windows (version 13.0). $P < 0.05$ was considered statistically significant.

RESULTS

Colonic damage

Macroscopic colonic damage: The results of assessment of gross and microscopic colonic injury are listed in Table 1. There was a minimal damage to the surface epithelium, and a mild inflammation of the mucosa but no transmural inflammation in the rats with an evident macroscopic respone to the treatment with melatonin. In the colitis group, the damage comprised broad mucosal ulcers with a surface layer of necrotic slough, accumulation of mesenteric fat, and fibrinous adhesions to the bowel. Macroscopic colonic damage score was the highest in colitis group (mean; 7.1) and was significantly lower in the colitis + melatonin group but higher than in the control group ($P < 0.01$).

Microscopic colonic damage: Histopathologic evaluation of microscopic colonic damage in the control group was in normal limits. Microscopic examination showed a low level of damage or inflammation in rats treated with melatonin (groups Ⅲ), whereas there was widespread destruction of the mucosa with transmural infiltration of neutrophils, monocytes, and lymphocytes in rats with colitis ($P < 0.05$) (Table 1).

Plasma TNF-α and endotoxin levels

Serum TNF-α and endotoxin levels in the colitis and colitis + melatonin groups were significantly increased on d 11 compared with the control group ($P < 0.01$, Figure 1). The increase in the colitis + melatonin group was significantly less than that in the colitis group ($P < 0.01$).

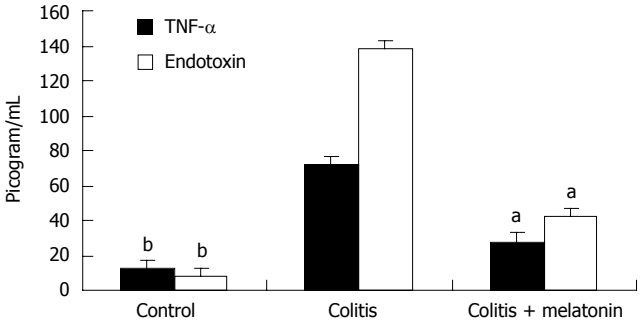


Figure 1 Serum TNF-α and endotoxin concentrations in rats. Vertical columns and error bars represent mean ± SD respectively. $^{b}P < 0.01$ *vs* colitis and colitis + melatonin groups, $^{a}P < 0.05$ *vs* colitis group.

Table 2 Incidence of bacterial translocation in different groups

Distant organ	Control[1]	Colitis[1]	Colitis + melatonin[1]	*P*
MLNs	0[b]	8	4[d]	< 0.01
Liver	0[d]	7	2[d]	< 0.01
Spleen	0[d]	6	2[d]	< 0.01
Portal blood	0[d]	6	1[d]	< 0.01
Systemic blood	0[d]	3	0[d]	< 0.01

MLNs: Mesenteric lymph nodes. Non-parametric Kruskal-Wallis test was used to compare bacterial translocation. [1]Number of positive tests for all animals tested (10 rats each group). $^{b}P < 0.01$ *vs* colitis and colitis + melatonin groups, $^{d}P < 0.01$ *vs* colitis group.

Bacterial translocation

In the control group, no bacterial growth was detected on d 11. TNBS-E colitis caused a significant increase in the frequencies of bacterial translocation in liver, spleen, MLNs, and portal blood. Compared with the colitis group, melatonin decreased the bacterial translocation in liver spleen, MLNs, and portal and systemic blood ($P < 0.05$). The incidence of bacterial translocation in different groups is shown in Table 2. Translocating bacteria were identified as Escherichia coli, Pseudomonas aeruginosa, Enterococus faecalis, and Staphylococcus aureus.

Myeloperoxidase levels in colon tissue

The colon MPO concentrations in rats are shown in Figure 2. The MPO levels in colonic tissue were higher in the colitis group than in the control and melatonin groups ($P < 0.001$). The colonic MPO activity was significantly lower in the melatonin treatment group than in the TNBS-treatment group ($P < 0.001$).

Caspase-3 levels

The caspase-3 level in colonic tissue was significantly higher in the TNBS-E treatment group than in the control group ($P < 0.001$). Treatment with melatonin significantly decreased the caspase-3 activity compared to the colitis group ($P < 0.01$, Figure 3).

DISCUSSION

Ulcerative colitis and CD are the 2 major clinical forms of IBD, clinically characterized by diarrheic attacks, weight loss, and abdominal pain. An increase in incidence has been noticed in the last decade, and the actual prevalence of IBDs is now estimated to be 150 to 200/100.000 people in Europe and North America[16].

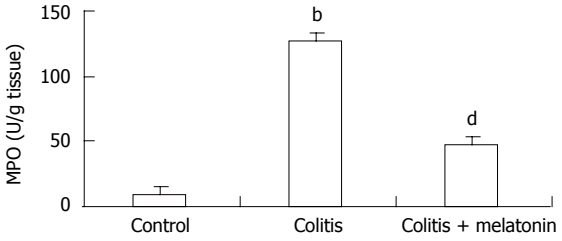


Figure 2 Colon MPO concentrations in rats. Vertical columns and error bars represent mean ± SD respectively. $^{b}P < 0.001$ *vs* control and colitis + melatonin groups, $^{d}P < 0.001$ *vs* colitis group.

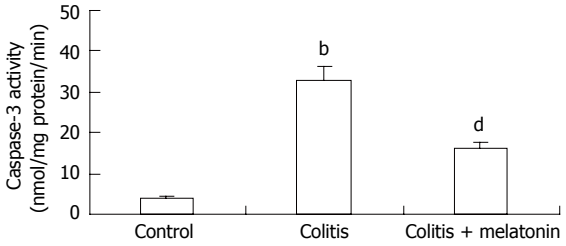


Figure 3 Colonic caspase-3 activity in all groups. Vertical columns and error bars represent mean ± SD respectively. $^{b}P < 0.001$ *vs* control group, $^{d}P < 0.01$ *vs* colitis group.

Although experimental animal models of colitis are of value in elicing some of the mechanisms of IBDs, the different models differ in their relative use[17]. Most animal models reported lack of certain characteristics of IBDs and model acute events rather than the chronic features typical of IBDs. TNBS colitis has been well characterized and has clinical, biochemical, and pathological similarities to colonic CD. It was reported that animals with TNBS colitis respond to drugs which are useful in treatment of IBDs[12]. This observation, together with the availability of a quantitative scoring system, makes it a useful system for the evaluation of new therapeutic agents. We, therefore, used TNBS-induced colitis, which has the dual advantage of chronicity and acuteness so often in patients with IBDs. Some other advantages of this model of colitis are its reasonable reproducibility and value in assessing the efficacy of therapeutic agents commonly used in the treatment of colitis[12,17].

The gastrointestinal tract is continuously exposed to toxins, microbes, microbial products, and other antigens. The intestinal epithelium has several important functions in protecting an organism from the potential deleterious effects of these agents acting as a physical barrier that limits the entry of luminal substances and microorganisms into the lamina propria[18]. Berg *et al*[19] have described bacterial translocation at the passage of viable bacteria through the epithelial mucosa into the lamina propria, and then to the MLNs and possibly other tissue. It was originally reported that bacterial translocation not only

means the migration of intestinal bacteria through the gut (the mechanisms and routes of which have never been identified) but also includes the consequences of this phenomenon, i.e., the infection of extraintestinal tissue with a subsequent systemic inflammatory response, possibly even leading to sepsis with multiple organ dysfunction[20]. The latter indicates that the immune system is no longer capable of eliminating migrating bacteria and/or inflowing toxins and that gut barrier dysfunction may be attributed to both morphological and functional disorders.

There appear to be three main mechanisms that promote bacterial translocation: impaired host defences, physical damage to the intestinal mucosa and disruption of the ecology of the indigenous gut flora resulting in intestinal bacterial overgrowth[19]. At least two of these factors are features of IBD, with an obvious physical disruption of the epithelial barrier by inflammation and ulceration and, as outlined above, disturbed bacterial defences with loss of communal stability and reduced colonization resistance[21]. Several human studies indicate that bacterial translocation also occurs in IBDs. Eade and Brooke[22] demonstrated that portal bacteremia and systemic bacteremia occur in 24% and 1% of patients being submitted to elective colectomy for ulcerative colitis, respectively. They also found that endotoxin plasma levels are positively correlated with clinical disease severity. In addition, bacteria were cultured in liver biopsies from 11% of these patients. Ambrose *et al*[23] reported that intestinal bacteria are cultured in the ileal serosa from 27% and in the MLNs from 33% of patients undergoing surgery for CD. In our study, both systemic and portal bacteremia were demonstrated, suggestingthat bacteria can be cultured in the MLNs, liver and spleen from TNBS-E colitis group. In this study, all the organ cultures were positive for enteric species, the extent of bacteria translocation from the colonic lumen correlated positively with the colon macroscopic and microscopic score, indicating that disruption of the physical barrier in this model is an important factor for promoting bacterial translocation.

It was previously demonstrated that the antioxidant melatonin exerts beneficial effects on different experimental colitis models of rats[5,6]. Mazzon *et al*[24] demonstrated that melatonin treatment significantly reduces the degree of colonic injury, proinflammatory cytokine release and apoptosis. However, the mechanism of the protective effects of melatonin has not been elucidated. The decreased free radical production, immunomodulatory effect and improvement in gut blood flow have to be considered[8]. Melatonin can protect gastrointestinal mucosa gainst damage by stimulating the immune system and fostering microcirculation and epithelial regeneration[25]. The involvement of melatonin in oxygen radical pathophysiology has been confirmed in different colitis models[26,27]. As reactive oxygen species play a significant role in the pathogenesis of colitis, melatonin may explain its protective effect on colonic inflammation. The present study showed that melatonin could exert protective effects on colonic inflammation and that reduction of bacterial translocation might be associated with the decreased cell apoptosis.

The extent of immune response can be assassed by measuring the level of MPO as a marker of neutrophil activity in the colon. In the present study, in all TNBS-E treated rats, colonic MPO activity was increased significantly compared to the control group, while melatonin treatment resulted in a decrease in this elevation. This finding is in agreement with earlier investigators who suggested a critical role of neutrophils in colitis[28]. Infiltration and activation of neutrophils lead to excessive generation of proinflammatory cytokines and reactive oxygen species that have been implicated in the pathogenesis of colitis[29]. The decrease in the expression of TNF-α in the melatonin-treated rats supported these findings. Melatonin is a direct antioxidant and also decreases free radical levels by stimulating the activities of enzymes involved in antioxidative defense. Melatonin prevents circulatory failure in rats with endotoxemia by inhibiting the release of TNF-α[30]. TNF-α is a multifunctional cytokine produced primarly by activated monocytes and macrophages and plays a crucial role in the initiation and continuation of mucosal inflammation and immunity[31,32]. TNF-α is involved in many cell processes including apoptotic cell death, metabolism, inflammation, thrombosis, and fibrinolysis[33] and induces the production of other cytokines including adhesion molecules, arachidonic acid metabolites, and activation of immune and non-immune cells[34]. It was reported that systemic inhibition of soluble TNF-α with infliximab (chimeric anti-TNF-α mAbs) induces remission in up to 50% of patients with CD and significantly improves clinical symptoms of most patients[35]. Worledge *et al*[36] reported that administration of TNF-α antibodies can effectively treat experimental rat colitis. In our study, TNF-α levels were parallel to the exacerbated inflammation and increased bacterial translocation in the colitis group. In contrast, TNF-α levels decreased in the melatonin group. At this point, TNF-α may be promising marker for monitoring IBDs activity and response to the treatment. Such findings indicate that blockade of TNF-α signaling has advantages in the treatment of IBDs. The results of our study also show the therapeutic potential of melatonin in human IBDs by affecting the TNF-α levels. In addition to its anti-inflammatory effect, melatonin action in the prevention of colitis may include stimulation of the immun system[37]. Although there is no direct evidence that the effect of melatonin on the immune response is enhanced in the gastrointestinal tract, indirect evidence indicates that melatonin administration to rats significantly increases the number and size of Peyer's patches[38].

Apoptosis, known as programmed cell death, is an essential physiological process required for the maintenance of tissue homeostasis. Insufficient or excessive cell death can contribute to disease. Although many factors are involved in apoptotic program, caspases have been shown to play a major role in the transduction of apoptotic signals[39]. Recent studies have also shown the induction of apoptosis in different cell lines in response to reactive oxygen species, peroxynitrite and nitric oxide[40]. In the present study, colonic caspase-3 activity was significantly higher in the TNBS-treated rats compared to the control group and treatment with melatonin significantly decreased the caspase-3 activity. Wang *et al*[41] found that melatonin inhibits hepatic caspase-3 activities and attenuates DNA fragmentation in D-galactosa-

mine-lipopolysaccharid-treated mice. Apoptosis may occur within several hours to several days after injury in some locations, thus, the suppression of cell death is clinically relevant. The role of apoptotic cell death in the inflamed intestine has not been clarified. In UC, the frequency of apoptosis is considerably increased[42] and loss of epithelial cells appears to occur mainly due to apoptosis[43]. Disregulation or inhibition of apoptosis appears to be important in cell proliferation, tissue hyperplasia, and malignant transformation of gastrointestinal epithelia[6]. These findings suggest that epithelial apoptosis may result in an alteration of the epithelial barrier function leading to pathogenic microorganism infiltration. Additonally, apoptosis regulates the lymphocyte population and may inhibit immune response at the inflammatory site[24]. Therefore, it has been proposed that an increased T-cell resistance to apoptosis may contribute to disease progression and mucosal alterations in CD[44]. These results indicate that the protective effects of melatonin against TNBS- induced colitis might be, at least in part, mediated by its anti-apoptotic effects.

In conclusion, melatonin reduces bacterial translocation in TNBS-induced colitis. The anti-inflammatory and anti-apoptotic effects of melatonin can reduce the extent of mucosal damage. Its similar effects in humans remain to be clarified.

COMMENTS

Background

Inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis have become important health problems in recent years. A growing body of data indicates that oxygen-derived free radicals and nitrogen species have been implicated as mediators of the disruption of the intestinal barrier in IBD, but their molecular targets and pathways have not been defined. It has been shown that the presence of endogenous melatonin in the gastrointestinal tract, and the melatonin's action as a scavenger of free radicals, have been implicated in the ameliorative effect on experimentaly-induced colitis.

Research frontiers

The epithelium of the colon consists of dynamic and rapidly proliferating cells that are profoundly affected by changes in luminal contents and injury. Previous experimental studies have shown that intrarectal instillation of 2, 4, 6-trinitrobenzene sulphonic acid (Sigma, St. Louis, Missouri) mixed with 30% ethanol (TNBS-E) promotes morphologic alterations in the colonic mucosa indicative of atrophy, increases apoptosis, and induces intestinal oxidative stress. Treatment with melatonin can attenuate bacterial translocation and apoptosis as shown in our study by the lower serum levels of tumor necrosis factor-α and endotoxin, the reduced colonic tissue myeloperoxidase and caspas levels.

Innovations and breakthroughs

This study demonstrated that melatonin reduced bacterial translocation in TNBS-induced colitis. The finding supports that exogenous melatonin exerts an anti-inflammatory activity. The mechanism of its anti-inflammatory effect is not entirely clear. In colitis, the frequency of apoptosis is considerably increased and loss of epithelial cells appears to occur mainly due to apoptosis. These results indicate that the protective effects of melatonin on TNBS-induced colitis might be, at least in part, mediated by its anti-apoptotic effects.

Applications

The results of the present study further demonstrate the important role of melatonin in regulating epithelial functions. The marked increase of bacterial translocation in postcolitis rats was reversed by melatonin administration due to its anti-inflammatory and anti-apoptotic effects. Further investigations are required to clarify whether melatonin is an effective and safe therapy for IBDs in human beings.

Terminology

Melatonin, the primary pineal hormone, is also synthesized from tryptophan and secreted in retina, salivary glands, thyroid, liver and intestine, and plays a fundamental role in the neuroimmuno- endocrine system. In the intestine, locally synthesized melatonin is released by enterochromaffin cells. It seems to act as a potent free-radical scavenger rendering it a strong antioxidant.

Peer review

The experiments are rational and reliable, the statistical methods used are appropriate, results show sufficient experimental evidence, and discussion is well organized and valuable.

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RAPID COMMUNICATION

Persistence of side population cells with high drug efflux capacity in pancreatic cancer

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Abstract

AIM: To investigate the persistence of side population (SP) cells in pancreatic cancer and their role and mechanism in the drug resistance.

METHODS: The presentation of side population cells in pancreatic cancer cell line PANC-1 and its proportion change when cultured with Gemcitabine, was detected by Hoechst 33342 staining and FACS analysis. The expression of ABCB1 and ABCG2 was detected by real-time PCR in either SP cells or non-SP cells.

RESULTS: SP cells do exist in PANC-1, with a median of 3.3% and a range of 2.1-8.7%. After cultured with Gemcitabine for 3 d, the proportion of SP cells increased significantly (3.8% ± 1.9%, 10.7% ± 3.7%, t = 4.616, P = 0.001 < 0.05). ABCB1 and ABCG2 expressed at higher concentrations in SP as compared with non-SP cells (ABCB1: 1.15 ± 0.72, 5.82 ± 1.16, t = 10.839, P = 0.000 < 0.05; ABCG2: 1.16 ± 0.75, 5.48 ± 0.94, t = 11.305, P = 0.000 < 0.05), which may contribute to the efflux of fluorescent staining and drug resistance.

CONCLUSION: SP cells with inherently high resistance to chemotherapeutic agents do exist in pancreatic cancers, which may be candidate cancer stem cells contributing to the relapse of the tumor.

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Key words: Side population cells; Hoechst 33342; Drug resistance; Cancer stem cells; Pancreatic cancer

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INTRODUCTION

Pancreatic cancer is one of the most aggressive malignancies and it is virtually incurable. Even for the patients who undergo potentially curative resection, the 5-year survival is less than 24% due to local recurrence and metastasis[1,2]. Emerging evidence has shown that cancer stem cells (CSCs), a small subset of cells within a tumor, are responsible for its malignancy[3]. So far, the significance of CSCs in hematological malignancy has been well known[4]. Although the presence and roles of these stem cells in solid tumors are poorly understood, their implication in breast carcinoma[5], brain cancers[6], prostate cancer[7] and lung cancer[8] are clear. As these cells must be eliminated in curative anti-cancer therapy, it is very important to identify and study CSCs.

Side population (SP) analysis could be used to identify CSCs[9]. SP cells can rapidly efflux lipophilic fluorescent dyes to produce a characteristic profile based on fluorescence-activated flow cytometric analysis[10]. Although representing only a small fraction of the whole cell population, they appear to be enriched in stem cells. Thus, they could provide a useful tool and a readily accessible source for cancer stem cell studies[11].

In addition to the application of SP cells in bone marrow obtained from patients with acute myeloid leukemia, which might be candidate leukemic stem cells[12], they have also been identified in various human solid tumors and cancer cell lines. Therefore, we presume that pancreatic cancer might also harbor SP cells, whose intrinsic dye efflux capacity is expected to be associated with the ability to export many cytotoxic drugs, hence increasing the risk of early relapse, and they may be candidate CSCs. We analyzed pancreatic cancer cell line PANC-1 and identified SP cells. The findings suggest that SP cells represent a previously unrecognized population of human pancreatic cancer cells.

Their high drug efflux capacity is related to the strong expression of drug-transporter proteins (including ABCB1 and ABCG2), which may be responsible for the malignancy of pancreatic cancer.

MATERIALS AND METHODS

Materials

Pancreatic cancer cell lines, PANC-1, were obtained from the Cancer Research Institute of the Chinese Academy of Sciences.

Hoechst, verapamil and propidium iodide (PI): Hoechst 33342 (Sigma), verapamil (Sigma) and PI (Sigma) were dissolved in distilled deionized water at 1 g/L, 5 mmol/L, and 1 g/L, respectively. All solutions were filter sterilized. Aliquots were kept frozen at -20℃. Hoechst and verapamil were allowed to thaw at room temperature protected from light before use. Under these conditions, the same aliquot could be re-frozen and re-used multiple times for Hoechst and up to five times for verapamil. PI was also thawed at room temperature and subsequently stored at 4℃.

DMEM+: Dulbecco's modified Eagle's medium (Gibco) containing 2% (v/v) fetal calf serum (Gibco) and 10 mmol/L HEPES (Gibco) can be stored up to 4 wk at 4℃.

HBSS+: Hanks' balanced salt solution (Gibco) containing 2% (v/v) fetal calf serum and 10 mmol/L HEPES can be stored up to 4 wk at 4℃.

PANC-1 medium: DMEM with 10% FBS (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin was stored at 4℃.

Gemcitabine (Eli Lilly) was dissolved in distilled deionized water at 1 g/L.

Primers of ABCB1, ABCG2 and β-actin were synthesized by Invitrogen Shanghai, China. Sequences of human primers used for real-time PCR experiments are shown in Table 1.

Table 1 Sequences of human primers used for real-time PCR experiments

Target mRNA	Primer sequence (5'-3')	Accession number	Size (bp)
ABCB1	5'AGGTTCCAGGATTGGCGTCTT3'	AF399931	156
	5'CCAGTCATTGCTGCGGTTTCA3'		1152-1307
ABCG2	5'AATACATCAGCGGATACTACAGAG3'	AY333756	179
	5'AGCCACCATCATAAGGGTAAACAT3'		1382-1560
β-actin	5'GAACGGTGAAGGTGACAG3'	NM_001101	293
	5'TAGAGAGAGTGGGGTGG3'		915-1207

Cell culture

The human pancreatic cancer cell line, PANC-1 was cultured as described above, and maintained in a 50 mL/L CO_2 atmosphere at 37℃ in a humidified incubator.

Hoechst 33342 and Pi dual-labeled dyeing

The cells, cultured to logarithmic phase, were prepared to single cell suspension in pre-warmed DMEM+. One g/L Hoechst 33342 was added to a final concentration of 5 mg/L, the cells were then incubated in the 37℃ water bath for exactly 90 min. The cells were centrifuged at 500 × *g*, 4℃ for 5 min and resuspended in 1-3 mL ice-cold HBSS+. Finally, the cells were counterstained with 2 mg/L PI for 5 min. The stained cells were then examined and photographed under an AX70 fluorescence microscope (Olympus, Orangeburg, NY).

Flow cytometry of SP cells

The SP was isolated with the technique described by Goodell *et al*[10]. The cells were cultured to logarithmic phase, then removed from the culture flask with trypsin and EDTA, washed, suspended at 10^6 cells/mL in pre-warmed DMEM+. The cells were then labeled in the same medium at 37℃ for 90 min with 5 mg/L Hoechst 33342 dye, either alone or in combination with 50 μmol/L verapamil (a calcium channel blocker and an inhibitor of some ABC transporters). Finally, the cells were counterstained with 2 mg/L PI to label dead cells.

Then, 3-5 × 10^4 cells were analyzed in a FACSVantage fluorescence- activated cell sorter (Becton Dickinson, Mountain View, CA) using a dual-wavelength analysis (blue, 424-444 nm; red, 675 nm) after excitation with 350 nm UV light. PI-positive dead cells (< 10%) were excluded from the analysis. The SP or non-SP cells were sorted.

Efflux and survival studies for cytotoxic drug gemcitabine

To examine whether increased drug efflux results in a survival advantage, 10^4 PANC-1 cells were cultured with or without Gemcitabine at a concentration of 5 mg/L for 3 d, and the percentage of SP cells was determined subsequently by the method as described above.

Real-time RT PCR

The SP cells or non-SP cells were harvested, RNA was isolated from them separately. Real-time RT-PCR was done to detect the expression of human ABCB1 and ABCG2, β-actin was used as a control. Total RNA was extracted from 10 samples of SP cells or non-SP cells each. In RT reaction process, 4 μL mRNA and 0.5 μL Oligo (dt) were added to 6.5 μL distilled water. After annealing for 5 min at 70℃ and immediate cooling on ice, 4.0 μm of 5 × first strand buffer, 2.0 μL of 10 mmol/L dNTP, 0.5 μL of RNasin and 0.5 μL RTase were added to get a total reaction volume of 20 μL. The reaction was allowed to proceed for 60 min at 37℃, followed by 5 min at 95℃ to inactivate the enzyme. In real-time PCR assay, each cDNA sample was repeated three times. The total PCR volume consisted of 1 μL cDNA, 1 μL SYBRGreen PCR I , 5 μL 10 × buffer, 1.6 μL primers, 7 μL $MgCl_2$, 0.5 μL Taq DNA Polymerase, 1 μL dNTP and 33 μL distilled water. After denaturation of the enzyme for 2 min at 94℃, the PCR assays were carried out for 45 cycles, with denaturation at 94℃ for 30 s, annealing at 57℃ for 30 s and extension at 72℃ for 30 s. Fluorometric PCR was performed with the FTC-2000 System. The level of expression of each transporter protein gene was determined relative to the β-actin gene.

Statistical analysis

All data were expressed as mean ± SD. Student's *t* test was used to determine changes in different groups. $P < 0.05$ was considered statistically significant.

RESULTS

Morphology of SP cells under fluorescence microscope

To observe the morphology of SP cells under fluorescence microscope, we stained PANC-1 with Hoechst 33342 and PI. As SP cells are able to expel fluorescent dye, they were Hoechst 33342 negative, which were colorless, while non-SP cells were Hoechst 33342 positive, which generated blue fluorescence (Figure 1).

Prevalence of SP cells in PANC-1

To determine the prevalence of SP cells in PANC-1, we stained 10 samples with Hoechst 33342 and PI, and analyzed them by flow cytometry. PANC-1 contained SP cells ranged from 2.1% to 8.7% in the total viable cells. The SP population was decreased greatly after treatment with verapamil, indicating that the populations were bona fide SP cells (Figure 2). Thus, pancreatic cancer cell line, PANC-1, contains SP cells, despite being maintained in culture for many years.

Efflux of gemcitabine from PANC-1 SP cells

To examine whether SP cells resist anti-cancer drugs more than non-SP cells do, we tested gemcitabine, which is usually used for the treatment of pancreatic cancer. The bulk cultures of PANC-1 cells were added 5 mg/L gemcitabine. By d 3, the proportion of SP cells increased (10.7% ± 3.7%, $t = 4.616$, $P = 0.001$, < 0.05), indicating the selection of this population (Figure 3).

Gene expression analysis

To investigate the association between SP phenotype and the expression of drug transporter genes, we isolated RNA from sorted SP and non-SP cells of PANC-1 and used a real-time RT-PCR assay to quantify the relative expression of two ABC transporter genes, ABCB1 and ABCG2. They were clearly expressed at higher concentrations in SP compared with non-SP cells (ABCB1: 1.15 ± 0.72, 5.82 ± 1.16, $t = 10.839$, $P = 0.000 < 0.05$; ABCG2: 1.16 ± 0.75, 5.48 ± 0.94, $t = 11.305$, $P = 0.000 < 0.05$) (Figure 4, Table 2).

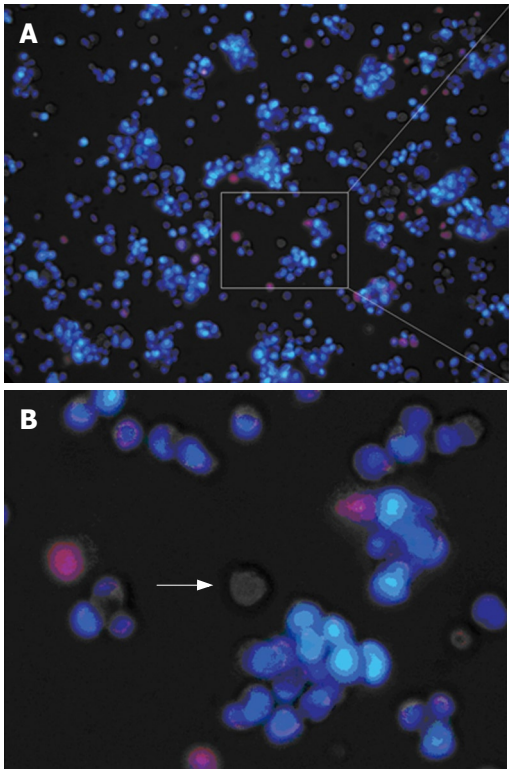


Figure 1 Hoechst 33342 and Pi dual-labeled dyeing.

DISCUSSION

The discovery of CSCs in solid tumors has changed our view of carcinogenesis and chemotherapy. In the cancer stem cell model, only a rare subset of CSCs drives tumor formation, the new therapeutic strategies should account for the potential differences in drug sensitivity or target expression between the tumorigenic cells and the more frequent non-tumorigenic cells. Therefore, to identify CSCs is very important. Up to date, only two general approaches have been adopted to identify and characterize CSCs: (1) Molecular markers, i.e. $CD44^+/CD24^-$ for breast CSCs[13], $CD44^+/alpha2\ beta1^{hi}/CD133^+$ for prostate CSCs[7], and $CD34^+/CD38^-$ for leukemia stem cells[14]; (2) SP isolated from tumors which has been proven to be an alternative to study CSCs, for they are enriched in CSC. For example, Chiba *et al*[15] found that SP cells in hepatocellular carcinoma cells possessed extreme tumorigenic potential and provided heterogeneity to the cancer stem cell system characterized by distinct hierarchy. As specific cell surface markers are largely unknown, SP analysis becomes a more useful tool for stem cell studies. In addition, malignant SP cells' intrinsic ability to expel anticancer drugs may directly improve their survival and sustain their clonogenicity during exposure to anticancer drugs, allowing disease recurrence when the therapy is withdrawn[16]. Identification of this tumor progenitor population might provide clues for improved therapeutic intervention.

Recently, SP cells have also been identified in various tumors and cancer cell lines, e.g. acute myeloid leukemia[12], neuroblastoma[17], melanoma[18], ovarian cancer[19], the C6 glioma cell line[20], human retinoblastoma cell line[21], human neuroblastoma cell lines[17], and various human gastrointestinal cancer cell lines[22]. However, no SP has been described in cell lines derived from Wilms' tumor, rhabdomyosarcoma or osteosarcoma[17]. To investigate the prevalence of SP cells in human pancreatic cancers, we stained PANC-1 with Hoechst 33342 dye and identified the SP based on its characteristic fluorescent profile in dual-wavelength analysis, as described above. PANC-1 contained SP cells ranging from 2.1% to 8.7% (median 3.3%) of the total viable cells.

Our study of the SP covers one of the most interesting areas in cancer biology, regarding carcinogenesis and drug resistance in the cancer therapy. Our findings suggest that SP cells in PANC-1 have an enhanced efflux capacity not only for Hoechst 33342 dyes but also for anti-neoplastic drugs. After 72 h exposure to Gemcitabine, the viability of the SP cells became markedly higher than that of the non-SP cells. It is the drug efflux capacity of SP cells that affords them a survival advantage. These indicate that SP cells play an important role in maintaining cancer foci,

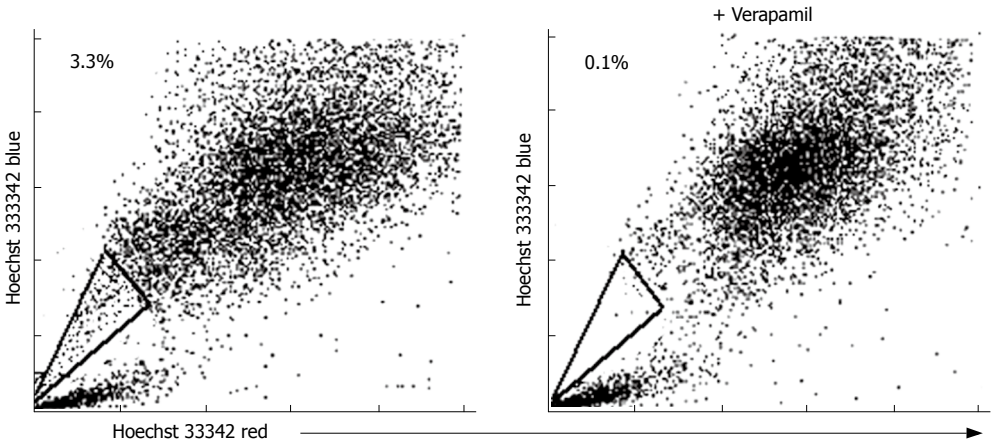


Figure 2 Existence of SP cells in PANC-1.

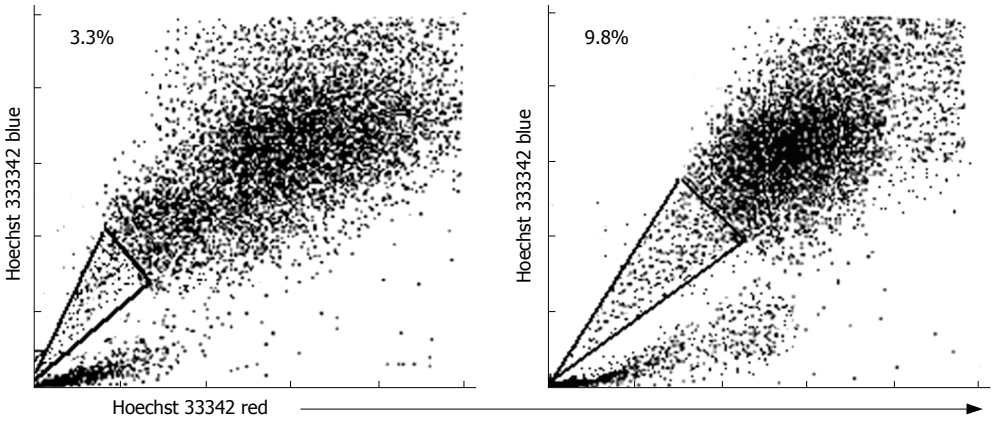


Figure 3 SP cells show a high resistance to anti-cancer-drugs.

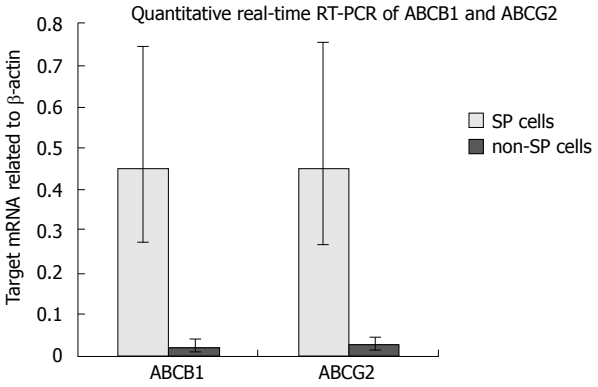


Figure 4 Relative expression of ABC transporter genes in PANC-1 SP and non-SP cells.

Table 2 Relative quantitation of mRNA using the $2^{-\Delta\Delta CT}$ method

	Target mRNA	ΔCT (Target mRNA-β-actin)	Target mRNA related to β-actin
non-SP cells	ABCB1	5.82 ± 1.16	0.0176 (0.0079-0.0393)
	ABCG2	5.48 ± 0.94	0.0224 (0.0116-0.0432)
SP cells	ABCB1	1.15 ± 0.72	0.4504 (0.2731-0.7431)
	ABCG2	1.16 ± 0.75	0.4483 (0.2662-0.7547)

which survive and proliferate after chemotherapy using their self-renewal and chemo-resistance capacities. They may be candidate pancreatic CSCs contributing to the relapse of the tumor.

The molecular mechanisms remain unclear that SP cells have intrinsic ability to expel dye and anticancer drugs. Goodell *et al*[10] demonstrated that multidrug resistance transporter 1 (MDR1), a member of ATP-binding cassette (ABC) transporter transmembrane proteins, may be involved in the efflux capacity of SP cells. But it cannot be taken as the single marker to identify and isolate SP cells, additional transporters should be analyzed. Zhou *et al*[23] demonstrated that ABCG2 has been shown to contribute to Hoechst 33342 efflux and produce an SP phenotype in a wild variety of tissues. Since SP is defined by ABC transporter activity, the expression of these transporters has been analyzed in various malignancies. For example, SP cells in a mouse glioma cell line and human neuroblastoma express high levels of ABCG2 and ABCA3[17], SP cells in hepatoma cell line, HuH7, express high levels of ABCG2 and ABCB1[22]. We also analyzed the expression of the ABC transporters in PANC-1, and found SP cells expressed higher levels of ABCB1 and ABCG2 than the non-SP cells. Therefore, ABCB1 and ABCG2 responsible for SP phenotype in pancreatic cancer could be speculated. However, some scholars proposed SP phenotype is not related to the presence of many "conventional" ABC transporter proteins such as MDR1, since MDR-1 knockout mice have normal numbers of SP cells[24]. So the

SP cells' intrinsic expel ability should be further studied.

As ABC transporters responsible for SP phenotype are implicated in drugs resistance and lower responses to cancer therapy[25,26], targeting the SP may offer an alternative option to improve drug resistance. Since ABC transporters are expressed in both normal and cancerous cells[27,28], they cannot be used to selectively target cancer cells unless specific strategies are applied. However, a whole genome DNA microarray analysis among normal SP cells, normal non-SP cells tumor SP cells and tumor non-SP cells, may be useful to identify potential candidates for specific CSCs markers, or even to define a CSCs pattern. The fact that CSCs specific surface markers are discovered, may permit isolation of this important population of cells, and revolutionize the cancer therapy and science[29,30].

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COMMENTS

Background

Pancreatic cancer is one of the most aggressive malignancies and it is virtually incurable. Over the past two decades, major advances in the understanding of this cancer have shifted to only increment of survival for the patients. Recent data suggested that cancers arising from rare stem cells are biologically distinct from their more numerous differentiated progeny, which may explain this paradox.

Research frontiers

This study focuses on the persistence of side population cells in pancreatic cancer and their role and mechanism in the drug resistance.

Innovation and breakthroughs

The difference from other related or similar studies is that, it is a new point of view to study drug resistance in pancreatic cancer.

Application

Identification of side population cells in pancreatic cancer might provide clues for improved therapeutic intervention. New drug development in pancreatic cancer should focus on this tumor progenitor population.

Terminology

Side population (SP) cells are cells which can rapidly efflux lipophilic fluorescent dyes to produce a characteristic profile based on fluorescence-activated flow cytometric analysis. Although representing only a small fraction of the whole cell population, they appear to be enriched in stem cells.

Peer review

This report demonstrates that SP cells with inherently high resistance to chemotherapeutic agents exist in pancreatic cancer and may be candidate cancer stem cells contributing to the relapse of the tumor.

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RAPID COMMUNICATION

Psychological intervention reduces postembolization pain during hepatic arterial chemoembolization therapy: A complementary approach to drug analgesia

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Abstract

AIM: To assess whether psychological intervention reduces postembolization pain during hepatic arterial chemoembolization therapy.

METHODS: Two hundred and sixty-two patients, who required hepatic arterial chemoembolization for hepatic malignancy and postembolization pain, were randomized into control group (n = 46, receiving medication) and intervention group (n = 216, receiving psychological intervention and medication in turn). The symptom checklist-90 (SCL-90) was used to scale the psychological symptoms of the patients before operation. Pain was scored with a 0 to 10 numeric rating scale (NRS-10) before and after analgesia as well as after psychological intervention (only in intervention group).

RESULTS: All psychological symptomatic scores measured with SCL-90 in the intervention group were higher than the normal range in Chinese ($P < 0.05$). The somatization, phobia and anxiety symptomatic scores were associated with pain numerical rating score before analgesia (r = 0.141, 0.157 and 0.192, respectively, $P < 0.05$). Patients in both groups experienced pain relief after medication, psychotherapy or psychotherapy combined with medication during the procedure ($P < 0.01$). Only some patients in the intervention group reported partial or entire pain relief (29.17% and 2.31%) after psychological intervention. The pain score after analgesia in the intervention group was significantly lower than that in the control group ($P < 0.01$).

CONCLUSION: Severe psychological distress occurs in patients with hepatic malignancy. Psychological intervention reduces pain scores significantly during hepatic arterial chemoembolization therapy and is thus, highly recommended as a complementary approach to drug analgesia.

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Key words: Liver neoplasms; Radiology; Psychological intervention; Non-pharmacologica; Pain

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INTRODUCTION

Hepatic carcinoma is one of the most common malignant visceral tumors in adults worldwide. Surgical resection remains the major treatment for hepatocellular carcinoma (HCC). However, only 8%-15% of patients meet the criteria for surgical resection[1-3] and the long-term results after resection are still unsatisfactory[4]. The liver is also the most frequent site of metastatic disease from extrahepatic primary tumors[5]. Resection of metastases can prolong the survival time of a small percentage of selected patients[6]. Transarterial chemoembolization (TACE), as a palliative therapy, is recommended for the treatment of unresectable hepatic neoplasms (primary and metastatic)[7,8]. The current routine technique of TACE is to inject iodized oil mixed with a single or a combination of chemotherapeutic agents into the neo-vasculature of hepatic neoplasms. The therapeutic goal is ischemic and cytotoxic insults to the tumor by occluding the feeding and collateral arteries of tumors. Right upper quadrant pain appears to be the most common complaint, followed by fidget, anxiety and noncompliance. Analgesia induced by drugs, such as intraarterial lidocaine and intravenous narcotic

analgesics, is required to alleviate the pain[9,10]. However, these medications have side effects and a few patients are not sensitive to sedative and analgesic medications. It is, therefore, necessary to develop non-pharmacologic approaches to improving the patient experience with painful procedures.

Non-pharmacologic practices, such as guided imagery, music therapy, hypnosis, and distraction, can effectively improve patient experiences during painful medical procedures, such as endoscopy[11]. There are few studies analyzing the effect of psychological intervention on pain suffered by hepatic cancer patients undergoing hepatic arterial chemoembolization therapy.

The aim of this investigation was to assess whether psychological intervention reduces postembolization pain during hepatic arterial chemoembolization therapy.

MATERIALS AND METHODS

Subjects

Between April 2002 and March 2006, 262 patients requiring hepatic arterial chemoembolization for hepatic malignancy (primary or metastatic) and postembolization pain agreed to participate in the study. The group comprised 186 men and 76 women with their age ranged from 13 to 76 years (mean age, 51.96 years). The diagnosis of hepatic tumor was confirmed by pathologic and histologic examination or elevated serum tumor markers. Patient exclusion criteria were as follows: (1) history of interventional diagnosis and/or therapy, (2) bellyache before embolization, (3) treatment with anxiolytic or narcotic analgesics 72 h prior to the therapy, (4) presence of encephalopathy or other significant alterations of mental status or cognitive impairment or visual and auditory deficits, (5) considerable cardiopulmonary morbidity.

Methods

Patients were randomized into control group (n = 46, receiving medication) and intervention group (n = 216, receiving psychological intervention and medication in turn). Their baseline characteristics, including demographic information (i.e., age and gender) and tumor type, were collected from the medical records.

The Chinese version of symptom checklist-90 (SCL-90), a psychiatric self-report inventory, was used to scale the psychological symptoms of the patients before operation. All patients were guided to answer 90 items with the uniform advices, and the 90 items in the questionnaire were scored on a five-point scale to reflect the psychological symptom patterns of patients. The items referred to the assessment of index for somatization dimensions, obsessive-compulsive tendencies, depression, anxiety, phobia, interpersonal sensitivity, hostility, paranoid ideations and psychotic states.

During the procedure, patients in the control group received medication when they experienced pain, whereas psychological intervention and drug analgesia were performed in turn on patients who were assigned to the intervention group for the postembolization pain. Patients in the control group and those with painful experience after psychotherapy in the intervention group received medications according to the digital subtraction angiograms and symptoms, e.g. antispasmodics for arterial spasm, narcotic analgesics for pain without characteristic signs and symptoms. The doses of medications were determined by the operator who performing the procedure according to the condition of patients. Psychological intervention was performed by two registered nurses with 7- and 10-year professional experience, respectively. Psychotherapy included encouragement, verbal suggestion, relaxation training, distraction with verbal communication on interest of the patients, therapeutic touch on face of the patients, guided imagery and intra-arterial placebo (0.5 mL of 0.9% sodium chloride injection). The pain was scored using a 0 to 10 numeric rating scale (NRS-10)[12] (0 = "no pain at all", 1-3 = "mild pain", 4-6 = "moderate pain", 7-9 = "severe pain", 10 = "unbearable pain") before and after analgesia, as well as after psychological intervention (only in intervention group).

Table 1 Baseline characteristics of patients

Characteristics	Control group (n = 46)	Intervention group (n = 216)	Significance	
Age (yr)	54.11 ± 10.85	51.50 ± 11.54	t = 1.41	P = 0.16
Gender				
Male	37	149		
Female	9	67	χ^2 = 2.42	P = 0.12
Tumor type				
Primary	27	135		
Metastatic	19	81	χ^2 = 0.23	P = 0.63

P > 0.05 for each comparison.

Statistical analysis

The data were analyzed with the Statistical Package for Social Sciences (SPSS 11.0 for Windows). The measurement data were expressed as mean ± SD and the enumeration data as proportions. Statistical tests used in bivariate analysis included χ^2 test and Student t test. The relationship between mental status and postembolization pain was evaluated with the Pearson correlation coefficient. $P < 0.05$ was considered statistically significant.

RESULTS

There were slight differences in baseline characteristics between the control and intervention groups and none of these differences was statistically significant (Table 1). No significant difference was found in psychological symptoms before the procedure between the two groups (Table 2). The psychological symptom scores in the intervention group were higher than the normal range in Chinese and the differences were statistically significant ($P < 0.05$) (Table 3).

Pearson correlation coefficients showed that the somatization dimensions and phobia were positively correlated with pain numerical rating score before analgesia in intervention group ($P < 0.05$). In addition, the anxiety level was also positively correlated with the pain numerical rating score before analgesia ($P < 0.01$) (Table 4).

Table 2 Psychological symptoms of patients measured with SCL-90 before the procedure (mean ± SD)

Psychological symptoms	Control group (n = 46)	Intervention group (n = 216)	Significance	
			t (or t')	P value
Somatization	1.55 ± 0.46	1.58 ± 0.44	0.42	0.68
Obsessive-compulsive tendencies	1.53 ±0.67	1.68 ± 0.61	1.49	0.14
Sensitivity	1.65 ± 0.57	1.58 ± 0.55	0.78	0.44
Depression	1.72 ± 0.50	1.64 ± 0.48	1.02	0.31
Anxiety	1.47 ± 0.40	1.51 ± 0.39	0.63	0.53
Hostility	1.41 ± 0.57	1.50 ± 0.60	0.93	0.35
Phobia	1.46 ± 0.38	1.40 ± 0.33	1.09	0.28
Paranoia	1.50 ± 0.49	1.42 ± 0.48	1.02	0.31
Psychosis	1.46 ± 0.30	1.41 ± 0.37	0.98	0.33

P > 0.05 for each comparison.

Table 3 Differences in psychological symptoms measured with SCL-90 in patients of intervention group and normal Chinese (mean ± SD) (Jin H *et al*, 1986)

Psychological symptoms	Intervention group (n = 216)	Norm (n = 7273)	Significance	
			t (or t')	P value
Somatization	1.58 ± 0.44	1.40 ± 0.40	5.94	< 0.01
Obsessive-compulsive tendencies	1.68 ± 0.61	1.49 ± 0.54	4.53	< 0.01
Sensitivity	1.58 ± 0.55	1.45 ± 0.52	3.61	< 0.01
Depression	1.64 ± 0.48	1.42 ± 0.49	6.51	< 0.01
Anxiety	1.51 ± 0.39	1.31 ± 0.42	6.91	< 0.01
Hostility	1.50 ± 0.60	1.37 ± 0.50	3.15	< 0.01
Phobia	1.40 ± 0.33	1.25 ± 0.40	6.54	< 0.01
Paranoia	1.42 ± 0.48	1.35 ± 0.49	2.07	< 0.05
Psychosis	1.41 ± 0.37	1.22 ± 0.37	7.44	< 0.01

Patients in both groups experienced pain relief after medication, psychotherapy or combined analgesia during the procedure. The pain scores after analgesic therapy were lower than those before analgesic therapy and the differences were statistically significant (P < 0.01) (Table 5).

Only some patients in the intervention group reported partial or entire pain relief (29.17% and 2.31%, respectively) after psychological intervention (Table 6).

The pain numerical rating scores for the control and intervention groups before analgesia showed no statistical difference, whereas the pain score after analgesia for the intervention group (psychological intervention and medication) was significantly lower than that for the control group (medication) (P < 0.01), indicating that the pain control was better in the intervention group than in the control group during the embolization procedure (Table 7).

Table 4 Correlation between psychological symptom scores (SCL-90) and pain numeric rating scores (NRS-10) before analgesia in intervention group (n = 216)

Psychological symptoms	Numerical rating score	
	Correlation coefficient (r)	P value
Somatization	0.141	< 0.05
Obsessive-compulsive tendencies	0.106	> 0.05
Sensitivity	0.097	> 0.05
Depression	0.031	> 0.05
Anxiety	0.192	< 0.01
Hostility	0.083	> 0.05
Phobia	0.157	< 0.05
Paranoia	0.124	> 0.05
Psychosis	0.08	> 0.05

Table 5 Pain score measured with numeric rating scale (NRS-10) during the procedure (mean ± SD)

	Numerical rating score	Significance[1]	
		t'	P value
Control group (n = 46)			
Pre-medication	4.35 ± 3.82		
Post-medication	1.64 ± 1.53	4.47	< 0.01
Intervention group (n = 216)			
Pre-psychotherapy	3.69 ± 2.46		
Post-psychotherapy	2.45 ± 1.96	5.79	< 0.01
Post-medication	0.29 ± 0.21	20.24	< 0.01

[1]Compared with pre-therapy NRS.

Table 6 Pain control with psychological intervention in intervention group (n = 216)

	n	%
Entire relief[1]	5	2.31
Partial relief[2]	63	29.17
Not relief	148	68.52

[1]Pain numerical rating scores (PNRS ⩽ 1) after psychotherapy; [2]PNRS ⩾ 3 before psychotherapy.

Table 7 Pain numerical rating scores (NRS-10) before and after analgesia: Comparison between control group and intervention group (mean ± SD)

	Control group (n = 46)	Intervention group (n = 216)	Significance	
			t'	P value
Pre-therapy	4.35 ± 3.82	3.69 ± 2.46	1.12	> 0.05
Post-therapy	1.64 ± 1.53	0.29 ± 0.21	7.3	< 0.01

DISCUSSION

TACE is an accepted palliative treatment modality for patients with primary HCC or hepatic metastases. If the tumor is unresectable, the patient is a poor surgical candidate. The clinical yield and survival rate in patients undergoing TACE have been proved[7,8,13]. More studies on TACE or combined methods, such as TACE combined with radiofrequency ablation, radiotherapy or percutaneous ethanol injection, have been reported recently[14-17]. In patients undergoing TACE, postembolization pain appears to be the most common complaint[2]. The pain usually occurs after embolization and peaks within 12-24 h. A large dose of opioid analgesics is often required in most patients when the pain is too severe to endure[2].

The etiology of pain after TACE is not well understood, but several theories have been postulated, including distention of the liver capsule, tumor necrosis, acute

ischemia of the liver parenchyma, and gallbladder ischemia secondary to inadvertent embolization of the cystic artery[2,18]. Infusion of iodized oil mixed with chemotherapeutic agents, in particular, results in acute, sharp right hypo-chondrium pain, followed by a deeper visceral pain, most likely due to swelling of the liver and stretching of the capsule that contains the nerve fibers[2]. It is believed that administration of narcotic analgesics can effectively control the pain during the procedure[9,10,19]. In our study, forty-six patients in the control group experienced a significant decrease in abdominal pain after drug analgesia ($P < 0.01$). However, the pain of most patients was not completely relieved after medication therapy.

In addition to physical suffering, psychiatric syndrome is increasingly recognized as a major component of distress in cancer patients[20-26]. Several factors, including the grief about current and anticipated losses, fear of death, concerns about loved ones and the effect of certain chemotherapeutic drugs on mood, *etc*[20-23], contribute to psychological distress[24]. Minagawa *et al*[27] and Godding *et al*[28] showed that about 30%-60% cancer patients have psychiatric disorders. Our study demonstrated that the SCL-90 factor scores for the intervention group were higher than those for the normal ($P < 0.05$), indicating that the psychological status is abnormal in patients with severe psychiatric disorders and hepatic carcinoma.

Psychological distress can result in systemic responses. Rorarius *et al*[29] performed a case-control study to evaluate the stress response and the sensitivity of clinical and biochemical stress markers in patients undergoing laparoscopically assisted vaginal or abdominal hysterectomy and showed that the most sensitive symptoms and markers of the systemic response are pain scores. Passik *et al*[25] surveyed 1109 cancer patients treated by 12 oncologists with Zung Self-Rating Depression Scale (ZSDS) and numerical rating scale, and found that the untreated psychological distress in cancer patients is associated with amplified pain. In our study, somatization, phobia and anxiety symptoms measured with SCL-90 were correlated with the postembolization pain ($P < 0.05$), which is consistent with the reported data[25].

Pariser *et al*[30] examined the effects of a telephone intervention in adults with arthritis and suggested that minimal intervention may produce positive changes in arthritis self-efficacy (ASE), depression, and pain in some adults. Other psychotherapies, e.g. hypnotic suggestion, relaxation training[31], distraction therapy[32], therapeutic touch[33,34], and imagery[35,36], are used in the treatment of disease-related pain. Our results reveal that psychological intervention decreased the pain score significantly ($P < 0.01$). However, only five patients (2.31%) reported entire pain control and most (68.52%) patients showed a slightly alleviated pain. Drug analgesia was still required by most patients in the intervention group during the procedure.

Several studies reported that psychological intervention during an invasive procedure, e.g. listening to music during endoscopy, could improve tolerance and compliance[37], alleviate pain[32] and decrease the dose of sedative drugs[38,39]. In our study, pain was well controlled after psychotherapy combined with medication, demonstrating that the pain control is better in the intervention group than in the control group ($P < 0.01$). The satisfaction of patients was increased accordingly.

In conclusion, patients with hepatic malignancy have severe psychological distress and psychological symptoms (i.e., somatization, phobia and anxiety) associated with the postembolization pain. Psychological intervention reduces pain scores significantly during hepatic arterial chemoembolization therapy and is highly recommended as a complementary approach to drug analgesia.

COMMENTS

Background

Postembolization pain is the most common complaint of patients during and after transarterial chemoembolization (TACE) for unresectable hepatic neoplasms. Drug analgesia is often required to decrease pain. However, these medications have side effects and a few patients are not sensitive to sedative and analgesic medications.

Research frontiers

Non-pharmacologic practices, such as guided imagery, music therapy, hypnosis, and distraction, can effectively improve patient experiences during painful medical procedures, such as endoscopy.

Innovations and breakthroughs

Psychological intervention can reduce pain significantly during hepatic arterial chemoembolization therapy.

Applications

Psychological intervention should be considered during hepatic arterial chemoembolization therapy.

Peer review

This is an interesting report on a complementary approach to drug analgesia during TAE for hepatic carcinoma.

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RAPID COMMUNICATION

Dissection of No. 13 lymph node in radical gastrectomy for gastric carcinoma

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Abstract

AIM: To evaluate the feasibility and safety of No. 13 lymphadenectomy in radical gastrectomy for gastric carcinoma.

METHODS: Medical records of the patients undergone No. 13 lymph node dissection during D2 gastrectomy for gastric carcinoma, were reviewed from March 2003 to May 2007.

RESULTS: One hundred and fifty-eight patients underwent No. 13 lymph node dissection for D2 gastric carcinoma, of them, 4 (2.53%) were found to have metastasis in No. 13 lymph node. Metastasis to No. 12 lymph node was detected in 6 patients and 4 of them had positive No. 13 lymph node. The operative morbidity except for wound infection was 15.19% (24/158), and hospital death rate was 1.27% (2/158). No obstructive jaundice caused by No. 13 lymph node metastasis after No. 13 lymph node dissection in radical gastrectomy for gastric carcinoma was detected during the follow-up study to end of January 2007.

CONCLUSION: Dissection of No. 13 lymph node in D2 gastrectomy for gastric carcinoma is safe with a low morbidity and mortality rate. Further study is needed to explore its long-term effect.

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Key words: Gastric carcinoma; Lymphadenectomy; Gastric carcinoma; No. 13 lymph node

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INTRODUCTION

Although gastric carcinoma tends to decline in its incidence, it still remains the most common cancer and the leading cause for cancer-related death in China[1-3]. D2 gastrectomy has been increasingly employed to treat patients with gastric carcinoma since 1990s in China[4,5]. In some medical centers, dissection of No. 12 lymph node has been performed in combination D2 gastrectomy for several years[5-7].

Regional recurrence and distant metastasis are the main causes for death of patients with gastric carcinoma after operation[8-10]. Metastasis from gastric carcinoma to retropancreatic nodes following radical gastrectomy can result in obstructive jaundice. We assume that we could prevent obstructive jaundice resulting from No. 13 lymph node metastasis if we dissect No. 13 lymph node in D2 gastrectomy for patients with gastric carcinoma. Therefore, the present study was to evaluate the results and feasibility of No. 13 lymphadenectomy in radical gastrectomy for gastric carcinoma.

MATERALS AND METHODS

One hundred and fifty-eight patients (75 males, 83 females, average age of 67 years, range 38-75 years) with gastric carcinoma, who underwent D13 lymph node dissection in combination with D2 lymphadenectomy from March 2003 to May 2007, were enrolled in this study. Tumor TNM staging was performed, showing stage I in 20 patients, stage Ⅱ in 71 patients, and stage Ⅲ in 67 patients. No. 13 lymphadenectomy was performed for tumors located in the lower third of the stomach. Coronary artery disease, chronic obstructive pulmonary disease, immunodeficiency disease, liver cirrhosis, and diabetes, and those at the age of over 75 years were excluded from the patients who were going to receive No. 13 lymph node dissection. Signs of distal lymph

Table 1 Complications of operation

	No of complication	No of death
Injury to CBD	3	1
Injury to spleen	4	0
Injury to portal vein	1	0
Injury to splenic vein	1	0
Injury to IVC	1	0
Pancreatic fistula	1	0
Duodenal fistula	1	0
Gastrojejunostomy fistula	3	0
Pulmonary infection	9	1

CBD: Common bile duct; IVC: Inferior vena cava.

node, liver, lung, and peritoneal metastasis were found both at pre-operation and at intra-operation.

D2 lymph node dissection was performed, followed by No. 13 lymph node dissection. The No. 13 lymph node dissection procedure consisted of incision of the lateral peritoneal attachment to duodenum, sharp dissection of duodenum and head of the pancreas from the inferior vena cava (IVC), exposure of posterior aspect of the pancreas. Subsequently 1-4 lymph nodes were removed. Reconstruction of digestive continuity was performed in a fashion of Billroth-Ⅱ. Complications were recorded prospectively. Hospital death was defined as within 30 d after surgery or during hospital stay longer than 30 d. The patients were followed up by regular clinic examination and telephone.

RESULTS

Among the 158 patients who underwent No. 13 lymph node dissection and D2 gastric carcinoma removal, 4 were positive for No. 13 lymph node with a positive rate of 2.53% (4/158). The metastasis to No. 12 lymph node was detected in 6 patients and 4 of them were positive for No. 13 lymph node. Injury to the spleen occurred in 4 patients and two of them underwent splenectomy during gastrectomy because of uncontrollable hemorrhage. Injury to the common bile duct was found in 3 patients and treated with placement of a T tube and choledochojejunostomy. Of these three patients, one developed peritonitis. The three patients had massive bleeding due to the injury to portal vein, splenic vein and inferior vena cava, respectively. Homeostasis was achieved with the stitch suture. After operation, one patient had pancreatic fistula, 3 patients had enteral fistula and two of them were managed conservatively. Nine patients had pulmonary infection and one of them developed acute respiratory distress syndrome (ARDS) and died finally. The overall operative morbidity was 13.92% (22/158) except for wound infection. The hospital death rate was 1.27% (2/158) (Table 1).

During the follow-up, no obstructive jaundice was found in patients undergoing radical gastrectomy with No. 13 lymph node dissected.

DISCUSSION

Surgical resection is the only curative treatment modality available for gastric carcinoma and radical lymph node dissection is an important part of curative resection[5,11]. Theoretically, removal of lymph nodes by extended lymph node dissection increases the chance for cure. However, the extent of nodal dissection is also a significant risk factor for complications and death[12,13]. It is generally accepted that D2 dissection is the standard procedure for patients who undergo surgery with curative intend, although there is a disagreement about the optimal extent of lymph node dissection.

The D2 gastrectomy procedure is known as "extended lymphadenectomy" in Western countries, while Japanese surgeons employ D2 as a standard technique, and use the term "extended" for para-aortic dissection[6,14-17]. The mortality of D2 gastrectomy is around 5% in Western countries, whereas it is less than 2% in Japan, and less than 1% in specialized institutions [15, 17-19]. Therefore, specialized surgeons can safely perform gastrectomy in combination with D2 lymphadenectomy for gastric carcinoma with a relatively low operative risk. Furthermore, some surgeons have performed D3 lymphadenectomy at gastrectomy for gastric carcinoma, which can remove all lymph nodes located in compartment 3[13,20]. Although complications of D3 lymphadenectomy are in an accepted range, the morbidity and mortality rates for D3 lymphadenectomy are significantly higher than those for D2 lymphadenectomy.

D2 dissection in gastrectomy, defined by the Japanese Research Society for Gastric Cancer, has been performed for gastric carcinoma since 1990s in China[4,6]. No. 13 lymph node dissection in gastrectomy for carcinoma is based on the follow-up studies showing metastasis of gastric carcinoma to No. 13 lymph node[4,6]. This is the reason why we performed No. 13 lymph node dissection for gastric carcinoma. In our series, complications (except for wound infection) occurred in 24 patients (15.19%) and the mortality rate was 1.27%, which is comparable to the reported data[15,17]. In our study, injury to the inferior vena cava was the only procedure-related complication and only one patient suffering from this kind of complications could be managed with the stitch suture, suggesting that extending the surgery to lymph nodes posterior to the pancreas does not increase hospital deaths.

Patients with co-morbid diseases are not fitted for surgery, such as those suffering from liver cirrhosis and ischemic heart diseases, two important risk factors for mortality[21,22]. Interestingly, studies from UK and Holland showed that splenectomy with or without distal pancreatectomy are highlighted as a major risk factor for operative morbidity and mortality[23,24]. The relatively low morbidity and mortality in our study may be due to the following factors: patients selected with good tolerance to the extending D2 lymphadenectomy, skillful/experienced surgeons performing the surgery[25], no need for splenectomy or pancreatectomy, and rational postoperative management.

In conclusion, No. 13 lymph node dissection in combination with D2 lymph node dissection in radical gastrectomy for gastric cancer can be performed with a low morbidity and mortality rate. Further study is needed to explore its long-term effect.

COMMENTS

Background

Gastric carcinoma is the most common cancer and the leading cause for cancer-related death in China. Up to now, operation is still the first choice of treatment. However, what is the appropriate extent of radical gastrectomy remains controversial.

Research frontiers

Most surgeons believe that D2 gastrectomy is the best procedure for most patients. However, metastasis of No. 13 lymph node has been found in patients with obstructive jaundice after gastrectomy. We studied the feasibility of No 13 lymphadenectomy in radical gastrectomy for gastric carcinoma and found that No. 13 lymph node dissection in combination with D2 lymph node dissection in radical gastrectomy for gastric cancer could be performed with a low morbidity and mortality rate.

Innovations and breakthroughs

In our study, No. 13 lymphadenectomy was safe and effective, showing that No13 lymphadenectomy in radical gastrectomy is feasible.

Applications

According to our study, it is safe and effective to perform No. 13 lymphadectomy in radical gastrectomy, which can prevent obstructive jaundice due to No. 13 lymph node metastasis.

Terminology

No. 13 lymphadenectomy means that dissecting lymph nodes around the head of pancreas during radical gastrectomy.

Peer review

This is a valuable record of a large series of extended gastrectomies for gastric cancer and, with amendments, worthy of consideration for publication.

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CASE REPORT

Pancreatic tuberculosis in a human immunodeficiency virus positive patient: A case report

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Abstract

Despite the increased incidence of tuberculosis related to human immunodeficiency virus (HIV) in recent decades, pancreatic tuberculosis has rarely been described. We report a case of pancreatic tuberculosis in a 39-year-old African man who presented with progressive dysphagia, vomiting, weight loss and productive cough, accompanied by localized epigastric pain and one episode of melena. HIV-1 testing was positive and lymphocyte subset profile showed CD_4 count of 9/mm^3. Abdominal computed tomography (CT) scan with contrast revealed a cystic mass in the body of the pancreas, significant portal and retroperitoneal cystic adenopathy, and multiple cystic lesions in the spleen and liver. CT guided cyst aspiration and node biopsy detected Mycobacterium tuberculosis. The patient responded well on antituberculosis and antiretroviral therapy. Tuberculosis rarely involves the pancreas, probably due to the presence of pancreatic enzymes which interfere with the seeding of Mycobacterium tuberculosis. Pancreatic tuberculosis is considered to be the result of dissemination of the infection from nearby lymphatic nodes. Endoscopic ultrasound or CT guided fine needle aspiration for cytology is the recommended diagnostic technique. Although the prognosis is good with anti-tuberculosis treatment, it could be fatal without correct diagnosis and treatment. The clinician's high index of suspicion of pancreatic tuberculosis and application of FNAB to obtain pathological evidence are extremely important to a correct diagnosis, especially in young HIV positive patients.

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Key words: Pancreatic tuberculosis; Human immunodeficiency virus infection; CT-guided fine needle biopsy

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INTRODUCTION

Subsequent to the AIDS epidemic, the incidence of tuberculosis has slowly increased in developed countries[1]. However, pancreatic tuberculosis is rarely described in the literature. We report a case of pancreatic tuberculosis with abdominal dissemination which presented with progressive dysphagia, weight loss and epigastric pain.

CASE REPORT

A 39-year-old African man presented with a six months progressive dysphagia, vomiting and 30 lb weight loss, productive cough for three weeks, accompanied by localized epigastric pain and one episode of melena. He had a history of positive PPD 4 years earlier with normal chest radiograph and had not received isoniazid prophylaxis. Physical examination revealed a 101 F temperature, marked pallor, sinus tachycardia, epigastric and right upper quadrant tenderness. Investigations showed a WBC count of 1.7×10^3/mm^3 (normal range, 4.8-10.8 $\times 10^3$/mm^3), anemia with hemoglobin at 5.1 g/dL (normal range, 14.0-18.0 g/dL), amylase 148 U/L (normal range, 30-110 U/L), lipase 29 U/L (normale range, 4-66 U/L) and LDH 551 U/L (normal range, 102-190 U/L). Chest radiograph, liver and renal function were normal. HIV-1 test was positive and lymphocyte subset profile showed CD_4 count of 9/mm^3. A barium swallow showed a normal esophagus. Abdominal ultrasound revealed a cystic pancreatic mass, lymph node enlargement and cholelithiasis. The blood, urine and sputum culture were negative. Abdominal computed tomography (CT) scan with contrast revealed a cystic mass in the body of the pancreas with significant portal and retroperitoneal adenopathy and multiple cystic lesions in the spleen and liver (Figure 1). CT guided cyst aspiration and node biopsy detected inflammatory cells, necrotic tissue and many AFB bacilli. PCR testing was positive for Mycobacterium tuberculosis. The patient clinically improved on antituberculosis medications: ethambutol, isoniazid,

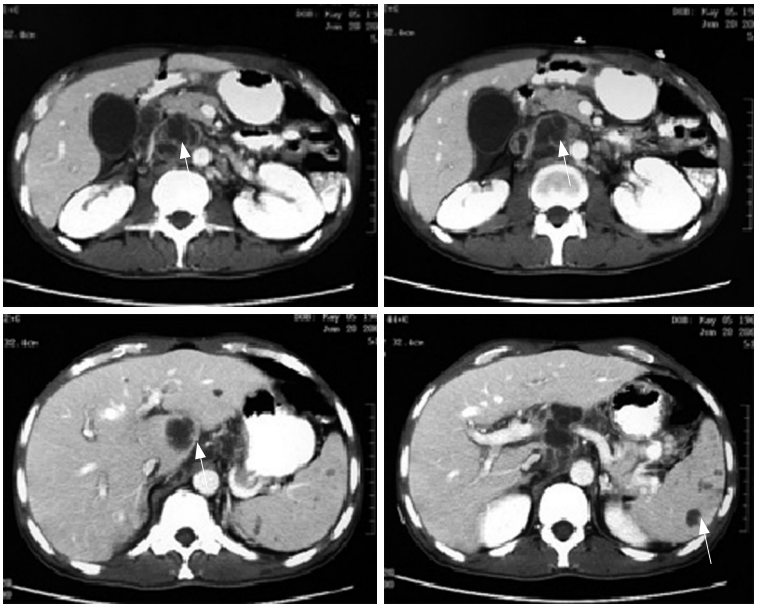


Figure 1 Abdominal CT with contrast showed cystic mass in the region of the body of pancreas and cystic lesions in the spleen and liver.

vitamin B6, pyrazinamide, rifampin and moxifloxacin. Five months later, a repeat ultrasound revealed resolution of the pancreatic cyst and adenopathy.

DISCUSSION

Tuberculosis uncommonly involves the pancreas, which is thought to be biologically protected from the infection of Mycobacterium tuberculosis. Probably, this is because the presence of pancreatic enzymes which interfere with the seeding of Mycobacterium tuberculosis[2]. Pancreatic tuberculosis is considered the result of the dissemination of the infection from near lymphatic nodes. A wide spectrum of symptoms has been reported. It ranges from abdominal pain, constitutional symptoms to such as weight loss, night sweat and anorexia, fever, obstructive jaundice[3,4], to massive gastro-intestinal bleeding[5] and iron deficiency anemia, pancreatic abscess[6]. In addition, acute pancreatitis[7], secondary diabetes[8] and chronic pancreatitis[9] have been also reported. Similar to the present case, the pancreatic cystic mass can mimic pancreatic cystic neoplasm[10,11]. Abdominal tuberculosis is independent of pulmonary disease in most patients, with the reported incidence of coexisting disease varying from 5% to 38%. Endoscopic ultrasound or computed tomography-guided fine-needle aspiration biopsy (FNAB) is recommended for diagnosis[12]. The prognosis is good with anti-tuberculosis treatment. However, it could be fatal without correct diagnosis and treatment[13]. Wide spectrum of symptoms and occurrence in the absence of chest disease make it difficult to diagnose. The clinician's high index of suspicion of pancreatic tuberculosis and application of FNAB to obtain pathological evidence are extremely important to a correct diagnosis, especially in young HIV positive patients.

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CASE REPORT

Familial occurrence of congenital bile duct dilatation

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Abstract

Congenital bile duct dilatation (CBD) that developed in a parent and son is presented. Familial occurrence of CBD is rare, with only a few male cases having been reported. Since the initial report of CBD occurring in siblings in 1981, a total of 20 cases (10 pairs) have been published as of 2007. Clinical and genetic features of CBD are discussed.

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Key words: Congenital bile duct dilatation; Familial occurrence; Choledochal cyst; Choledochocele; Anomalous pancreaticobiliary duct junction

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INTRODUCTION

Choledochal cyst is a rare congenital dilatation of the extrahepatic and/or intrahepatic biliary tract. Some possibilities have been postulated regarding inheritance of congenital bile duct dilatation (CBD), since several familial CBD cases have been reported. However, the etiology of CBD is essentially unknown. In this report, a case of familial occurrence of CBD is presented along with a review of the literature.

CASE REPORT

Case 1 was a 65-year-old woman, the mother of case 2, who was admitted to Matsusaka City Hospital, Japan, complaining of back pain and fever. Serum amylase level was normal. Liver function impairment was noted: aspartate aminotransferase (AST) 216 U/L, alanine aminotransferase (ALT) 228 U/L, and total bilirubin 1.3 mg/dL. A CT scan revealed a cystic dilatation of the common bile duct. With the diagnosis of choledochal cyst, the patient underwent cholecystectomy, resection of the dilated bile duct, and hepaticojejunostomy. Intraoperative cholangiopancreatography showed extrahepatic bile duct dilatation and an anomalous pancreaticobiliary duct junction (APBDJ) (Figure 1A and B). Amylase level in the bile was 4735 U/L. Microscopic examination of the resected specimen revealed no evidence of malignancy. The postoperative course was uneventful.

Case 2 was a 25-year-old man, the son of case 1, who was admitted to Mie University Hospital, Japan, with complaints of epigastric pain and fever. Amylase level in the serum and urine was 2639 and 56 720 U/L, respectively. Endoscopic retrograde cholangiopancreatography (ERCP) showed cystic dilatation of the extra- and intrahepatic biliary ducts associated with APBDJ (Figure 2). With the diagnosis of choledochal cyst, cholecystectomy, excision of the dilated bile duct, and hepaticojejunostomy were performed. Amylase level in the bile was 138 700 U/L. No malignancy was found histologically in the resected specimen. The patient was discharged without any complication.

DISCUSSION

Congenital choledochal cyst is a relatively rare disease and its incidence is between 1 in 130 000 and 1 in 2 million[10]. There is a female predominance (ratio 1:3)[11,12] and the lesions are more common in Asians, with the majority of reported cases in Japan[11]. Choledochal cysts usually present in infancy or childhood, which supports a congenital origin. Although anomalous pancreaticobiliary duct junction (APBDJ) is thought to be an essential causative factor of CBD, the etiology of CBD remains unclear. Since Chiba *et al* first described in 1981 cases of CBD occurring in

Table 1 Reported cases of familial occurrence of congenital bile duct dilatation

Year	Authors	Combination	Age	Extrahepatic bile duct	Intrahepatic bile duct dilatation	Anomalous pancreaticobiliary duct junction (APBDJ)
1981	Chiba[1]	Elder sister	4 yr 10 mo	Fujiform	+	+
		Younger sister	3 yr 9 mo		+	+
1985	Iwama[2]	Mother	48 yr	Fujiform	-	+
		Daughter	11 yr	Cystic	-	unknown
1986	Akiyama[3]	Mother	34 yr	Cystic	Unknown	unknown
		Daughter	3 mo		+	unknown
1987	Date[4]	Elder brother	7 yr	Cystic	-	+
		Younger sister	10 yr	Fujiform	-	unknown
1990	Iwafuchi[5]	Mother	25 yr	Cystic	+	+
		Daughter	2 yr 7 mo	Fujiform	-	+
1996	Narita[6]	Mother	48 yr	Cystic	Unknown	+
		Daughter	34 yr		+	+
1998	Iwata[7]	Mother	33 yr	Choledochocele	-	-
		Daughter	7 d	Cystic	Unknown	+
1999	Lane[8]	Dizygotic twins	3 yr	Cystic	-	+
		(Females)	3 yr	Fujiform	+	+
2005	Tokuhara[9]	Dizygotic twins	2 yr 4 mo	Fujiform	+	+
		(Females)	2 yr 6 mo	Cystic	-	+
2002	Our case	Mother	65 yr	Cystic	-	+
		Son	25 yr		+	+

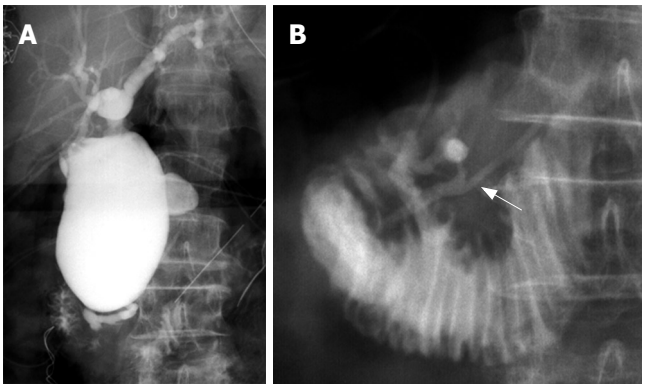


Figure 1 **A**: Intraoperative cholangiopancreatography, showing cystic dilatation of the common bile duct; **B**: Intraoperative cholangiopancreatography, showing an APBDJ (arrow).



Figure 2 ERCP showing dilatation of the extra- and intrahepatic bile ducts, as well as an APBDJ (arrow).

siblings[1], a total of 20 cases (10 pairs), including ours, of familial occurrence have been reported (Table 1). Eighteen patients (90%) were women and only two (10%) were men. Of these 10 pairs, four were siblings and six were parent and child. APBDJ was noted in 15 cases (75%). In addition, two pairs (20%) were dizygotic twins who both developed CBD. As for genetic background of CBD, Iwama *et al* have postulated two possible patterns of inheritance: an X-linked dominant trait or an autosomal dominant trait with relatively low penetrance in males[2]. The present case of CBD with APBDJ suggests a maternally inherited condition, i.e. an X-linked dominant trait. Besides our literature review, a total of six pairs of monozygotic twins have been described in which CBD appeared in one of each pair of twins[13-16]. The phenotypic discordance in monozygotic twins cannot be explained only by inheritance. Other factors, such as acquired or environmental conditions, might have a role in the development of CBD. To clarify the etiological basis of CBD, it will be necessary to accumulate more familial cases of CBD.

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CASE REPORT

Three cases of solitary Peutz-Jeghers-type hamartomatous polyp in the duodenum

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Abstract

A solitary Peutz-Jeghers-type hamartomatous polyp in the duodenum is rare, and few reports have described its characteristic endoscopic features. We describe three cases of solitary Peutz-Jeghers-type hamartomatous polyp and their endoscopic findings in detail. The polyp in all of our three cases showed an irregularly lobular or nodular surface, whereas adenomas often show a regularly nodular or granular surface. The color of the polyp was whitish in all of our cases. In the present cases, close observation by endoscopy revealed that the solitary Peutz-Jeghers-type hamartomatous polyps looked whitish because of the presence of diffusely scattered white spots on the surface of the polyps. Duodenal polyps that exhibit the aforementioned endoscopic characteristics may be diagnosed as Peutz-Jeghers-type hamartomatous polyps and treated by polypectomy because of the malignant potential.

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Key words: Duodenal polyp; Hamartoma; Peutz-Jeghers polyp

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INTRODUCTION

Peutz-Jeghers syndrome is a rare, autosomal-dominant disorder characterized by hamartomatous polyps in any part of the alimentary tract and mucocutaneous pigmentation[1]. Recently, a patient with a Peutz-Jeghers polyp without either mucocutaneous pigmentation or a positive family history was described as a case of solitary Peutz-Jeghers-type hamartomatous polyp. It has been suggested that this condition should be considered as a clinical entity different from Peutz-Jeghers syndrome[2]. A solitary Peutz-Jeghers-type hamartomatous polyp is histologically characterized by tree-like branching of smooth muscle fibers, with a core of smooth muscle, covered by mucosal tissue of near-normal appearance[3]. A solitary Peutz-Jeghers-type hamartomatous polyp in the duodenum is rare, and few reports have described its characteristic endoscopic features[2,4-10]. We describe three cases of solitary Peutz-Jeghers-type hamartomatous polyp and their endoscopic findings in detail. The polyp in patient 2 was unique in that a focus of adenocarcinoma was present within a solitary Peutz-Jeghers-type hamar-tomatous polyp.

CASE REPORT

Patient 1 was a 59-year-old woman. She visited our hospital complaining of epigastric discomfort. An upper gastrointestinal barium meal study revealed a polyp, 18 mm × 15 mm in diameter, in the second portion of the duodenum. Upper gastrointestinal endoscopy revealed a semi-pedunculated polyp on the oral side of the Papilla of Vater (Figure 1). Histological examination of a biopsy specimen obtained from the lesion showed hyperplastic duodenal mucosa, suggestive of the diagnosis of a hyperplastic polyp. Surgical resection of the polyp was performed under informed consent. Histologically, the polyp consisted of branching bundles of smooth muscle fibers covered by hyperplastic duodenal mucosa (Figure 2).

Patient 2 was a 68-year-old woman. She visited our hospital because of epigastralgia. Upper gastrointestinal endoscopy revealed a peduncular polyp, 10 mm × 8 mm in diameter, in the second portion of the duodenum (Figure 3). Histological examination of a biopsy specimen obtained from the lesion revealed findings suggestive of an adenoma. Endoscopic polypectomy was performed. Histologically, the polyp consisted of branching bundles of smooth muscle fibers covered by hyperplastic duodenal mucosa containing a focus of cancer near the surface (Figure 4).

Patient 3 was a 60-year-old woman. Upper gastro-intestinal endoscopy as part of a health checkup revealed a semi-pedunculated polyp, 10 mm × 10 mm in diameter, in the duodenal bulb (Figure 5). Histological examination

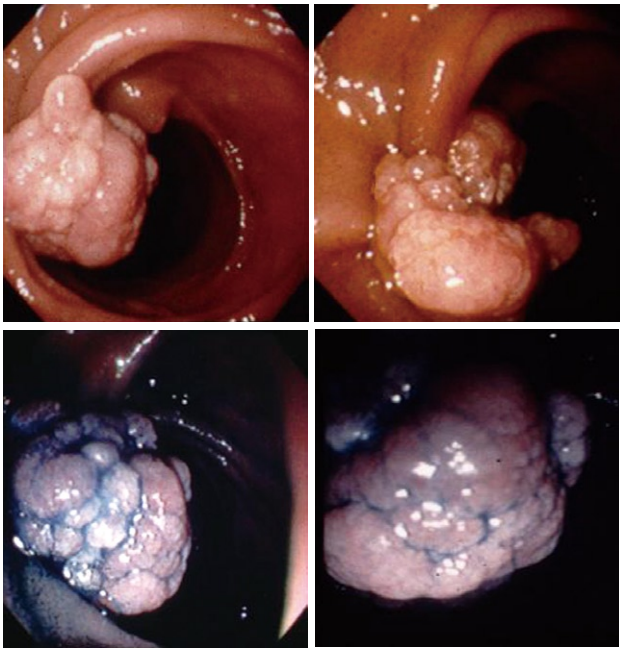


Figure 1 Upper gastrointestinal endoscopy in patient 1 revealed a semi-pedunculated polyp, 18 mm × 15 mm in diameter, on the oral side of the Papilla of Vater. The polyp showed an irregularly lobular surface and slightly whitish color with diffusely scattered white spots on the surface.

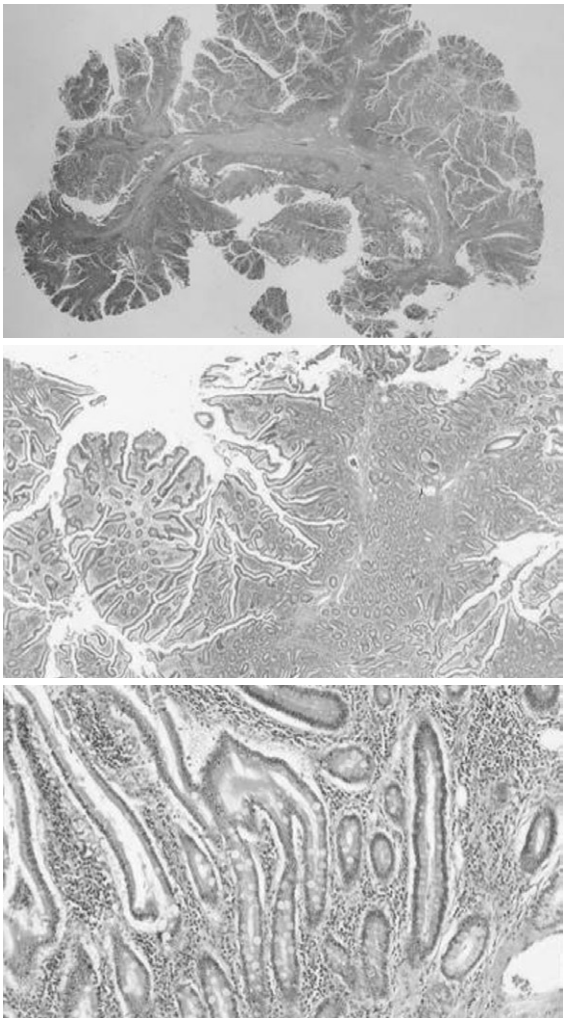


Figure 2 Histologically, the polyp consisted of branching bundles of smooth muscle fibers covered by hyperplastic duodenal mucosa.

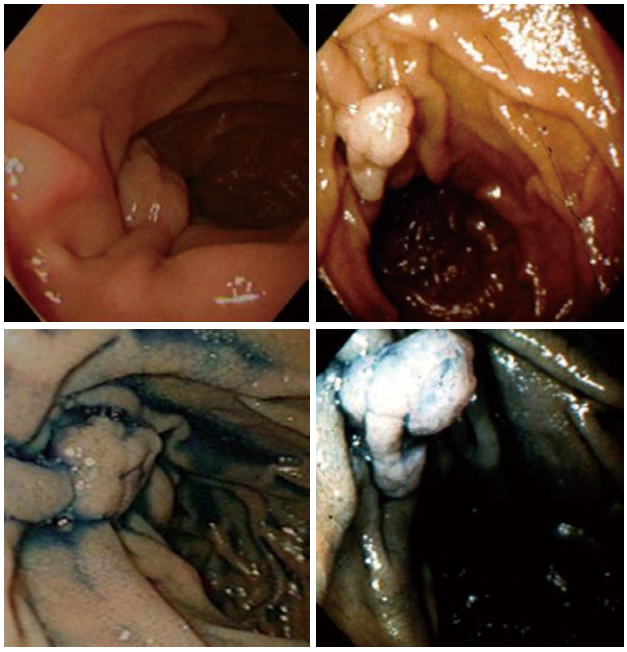


Figure 3 Upper gastrointestinal endoscopy in patient 2 revealed a peduncular polyp, 10 mm × 8 mm in diameter, in the second portion of the duodenum. The polyp showed an irregularly lobular surface and slightly whitish color with diffusely scattered white spots on the surface.

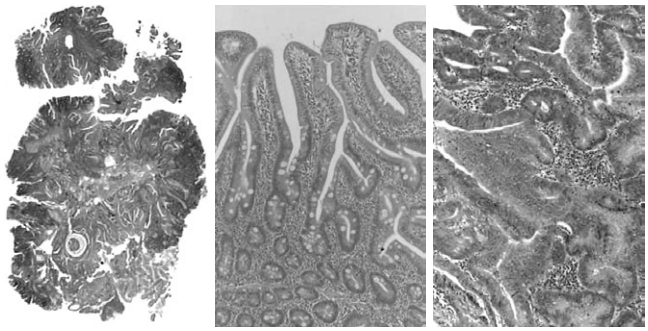


Figure 4 Histologically, the polyp consisted of branching bundles of smooth muscle fibers covered by hyperplastic duodenal mucosa containing a focus of cancer near the surface.

of a biopsy specimen obtained from the lesion showed hyperplastic duodenal mucosa. Endoscopic polypectomy was performed. Histologically, the polyp consisted of branching bundles of smooth muscle fibers covered by normal duodenal mucosa. Brunner's glands existed in the central part of the polyp (Figure 6).

All of the polyps showed an irregularly lobular or nodular surface and slightly whitish color with diffusely scattered white spots on the surface (Figures 1, 3 and 5). None of the three patients showed mucocutaneous pigmentation or a history of familial polyposis. We found no other polyps on colonoscopy or small-bowel follow-through examination with barium in any of the three patients.

DISCUSSION

A MEDLINE search of the English language literature revealed only nine well-documented cases of a solitary Peutz-Jeghers-type hamartomatous polyp of the

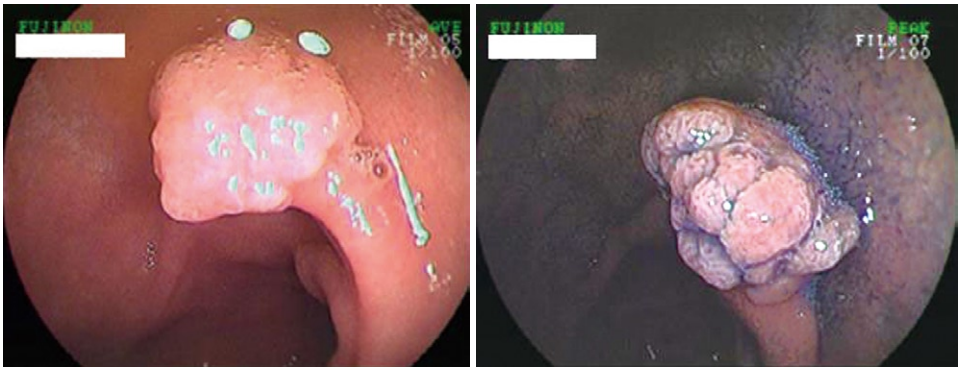


Figure 5 Upper gastrointestinal endoscopy in patient 3 revealed a semi-pedunculated polyp, 10 mm × 10 mm in diameter, in the duodenal bulb. The polyp showed an irregularly nodular surface and slightly whitish color with diffusely scattered white spots on the surface.

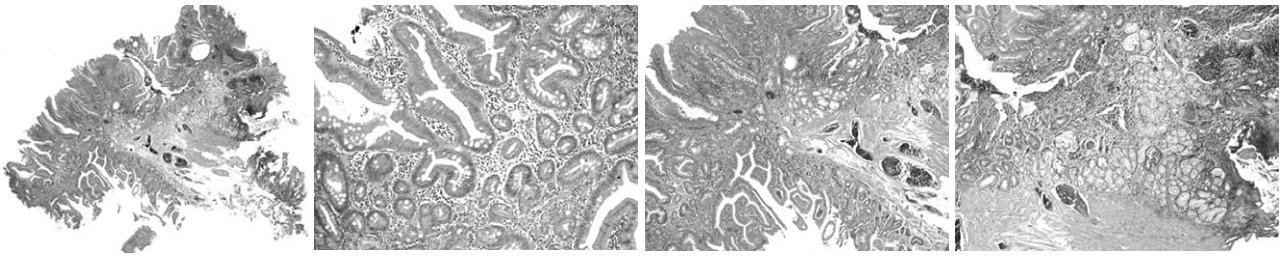


Figure 6 Histologically, the polyp consisted of branching bundles of smooth muscle fibers covered by normal duodenal mucosa. Brunner's glands existed in the central part of the polyp.

Table 1 Endoscopic finding

No	Author	Surface	Color	Biopsy	Therapy	Malignancy
1	Bott	Not described	Not described	Inflamation	Surgery	-
2	Naitoh	cerebriform	Not described	Not described	Endoscopic polypectomy	-
3	Tanaka	Lobrated	Not described	Not described	Endoscopic polypectomy	-
4	Tanaka	Multi lobrated	Reddish	Benign	Endoscopic polypectomy	-
5	Nerbi	Lobrated	Not described	Lymphoplastic	Surgery	-
6	Ichiyoshi	Lobrated or fine granular	Normal	Non neoplastic	Endoscopic polypectomy	○
7	Kitaoka	Lobrated	Not described	Not described	Endoscopic polypectomy	-
8	Itaba	Not described	Normal	Normal	Endoscopic polypectomy	-
9	Itaba	Lobrated	Normal	Normal	Endoscopic polypectomy	-
10	Our case1	Lobrated or nodular	Whitish	Hyperplastic polyp	Surgery	-
11	Our case2	Lobrated or nodular	Whitish	Adenoma	Endoscopic polypectomy	○
12	Our case3	Lobrated or nodular	Whitish	Hyperplastic polyp	Endoscopic polypectomy	-

duodenum (Table 1)[2,4-9]. Few of these reports have described the endoscopic characteristics of these polyps in detail. The polyp in each of our three cases showed an irregularly lobular or nodular surface, whereas adenomas often show a regularly nodular or granular surface; this finding was compatible with previous reports[2,5-9]. The color of the polyp was whitish in all of our cases, whereas previous reports have described the polyps to be red to normal color[6,7,9]. In the present cases, close observation by endoscopy revealed that the solitary Peutz-Jeghers-type hamartomatous polyps looked whitish because of the presence of diffusely scattered white spots on the surface of the polyps as Sugimoto *et al* reported[10]. We could not confirm the diagnosis by endoscopic biopsy in any of our cases because the polyps were covered by mucosal tissue of near-normal appearance. We consider that duodenal polyps that exhibit the aforementioned endoscopic characteristics may be diagnosed as Peutz-Jeghers-type hamartomatous polyps.

The polyp in patient 2 was unique in that a focus of adenocarcinoma was present within the solitary Peutz-Jeghers-type hamartomatous polyp and histological examination of a biopsy specimen revealed findings suggestive of an adenoma. Hamartomatous polyps are generally considered to have very low malignant potential[11]. However, some reports described areas of neoplastic change, such as adenomatous or carcinomatous change in 3%-6% of the polyps in Peutz-Jeghers syndrome[12,13].

Ichiyoshi *et al* also reported a case of solitary Peutz-Jeghers-type hamartomatous polyp of the duodenum containing a focus of adenocarcinoma[7]. Therefore, because of the malignant potential, a solitary Peutz-Jeghers hamartomatous polyp of the duodenum should be removed.

In conclusion, a solitary Peutz-Jeghers-type hamartomatous polyp of the duodenum is endoscopically characterized by a lobular or nodular surface and whitish color with diffusely scattered white spots on the surface. Duodenal polyps that exhibit the aforementioned endoscopic characteristics may be diagnosed as Peutz-Jeghers-type hamartomatous polyps and treated by polypectomy because of the malignant potential.

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CASE REPORT

Toothpick impaction with sigmoid colon pseudodiverticulum formation successfully treated with colonoscopy

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Abstract

Foreign bodies in the colon are encountered with increasing frequency, but only sporadic reports concerning their management have appeared in the literature. While most ingested foreign bodies usually pass through the gastrointestinal tract uneventfully, sharp foreign bodies such as toothpicks infrequently cause intestinal perforation and may even result in death. We report our experience with a patient with a sigmoid colon pseudodiverticulum formation, a complication of accidental ingestion of a toothpick that was diagnosed and successfully managed colonoscopically.

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Key words: Toothpick; Colon; Colonoscopy; Pseudodiverticulum

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Chung YS, Chung YW, Moon SY, Yoon SM, Kim MJ, Kim KO, Park CH, Hahn T, Yoo KS, Park SH, Kim JH, Park CK. Toothpick impaction with sigmoid colon pseudodiverticulum formation successfully treated with colonoscopy. *World J Gastroenterol* 2008; 14(6): 948-950
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INTRODUCTION

If an ingested foreign body successfully navigates the esophagus, it will frequently pass through the entire gastrointestinal tract. Opposition to passage beyond the esophagus, however, can be caused by anatomic conditions or factors specific to the foreign body. Impaction generally occurs at sites of narrowing, such as the pylorus, the ligament of Treitz, the ileocecal valve, or the rectosigmoid junction[1]. Furthermore, long, narrow, or pointed foreign bodies are associated with a higher risk of impaction, as well as complications such as perforation.

We describe here a case of abdominal pain caused by a toothpick impacting the sigmoid colon, which resulted in a pseudodiverticulum formation. The foreign body was diagnosed and managed successfully with colonoscopy. To our knowledge, colonic pseudodiverticulum caused by toothpick impaction has not been reported.

CASE REPORT

A 52-year-old man presented with lower abdominal pain of 1-mo duration. The pain began in the epigastric area and migrated to the lower abdominal area. He had a history of traumatic subdural hematoma 3 mo ago and has been treated with an anticonvulsant drug. He was not a smoker and his use of alcohol was described as only social. Physical examination revealed mild lower abdominal tenderness without rebound tenderness. Laboratory and endoscopic examinations were recommended, but he refused further evaluation. Seven days later, he revisited our hospital with severe lower abdominal pain. On physical examination, aggravated tenderness was noted in the lower abdominal region without rebound tenderness. Laboratory examination revealed only mild leukocytosis of 11 000/mm^3 with elevated C-reactive protein (38.90 mg/L). The next day, colonoscopy was performed and revealed a fixed sigmoid loop with a toothpick of 6 cm length that had impacted the distal sigmoid colon with surrounding mucosal erythema and edema. The toothpick was cautiously removed using foreign-body extraction forceps (Figure 1A). There were some yellowish discharge and hyperemia on the surface of impacted colonic mucosa, but no bleeding was observed (Figure 1B). Subsequent questioning yielded a history of accidental toothpick ingestion during an afternoon nap 2 wk ago. Abdominal CT revealed severe wall thickening, pericolic fat infiltration and peritoneal thickening in the distal sigmoid colon. In addition, a small air-containing cavity lined by thin epithelium was noted in the serosal surface of the distal sigmoid colon, which was consistent with pseudodiverticulum (Figure 2). There was no evidence

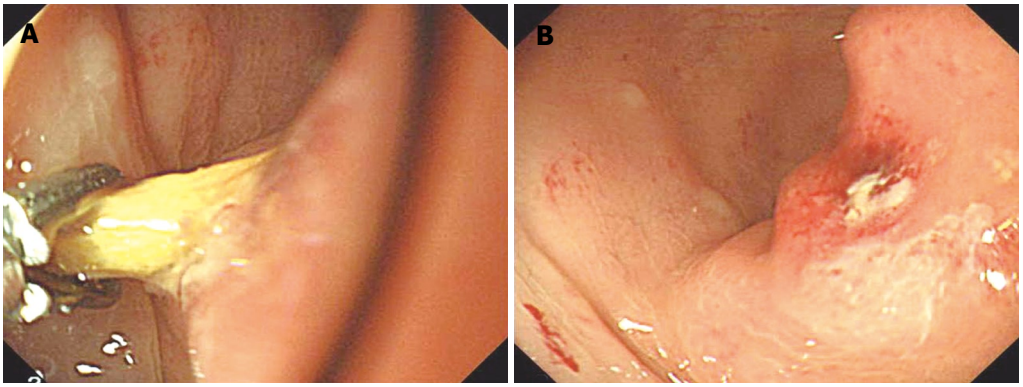


Figure 1 **A**: The endoscopic appearance of the toothpick in the sigmoid colon and removal of the toothpick was achieved using foreign-body extraction forceps. **B**: The endoscopic appearance of impacted colonic mucosa after removal of toothpick.



Figure 2 Initial abdominal CT shows severe wall thickening, pericolic fat infiltration and peritoneal thickening in the distal sigmoid colon. In the serosal surface, a small air-containing cavity lined by thin epithelium (arrow) is noted, which is consistent with a pseudodiverticulum.



Figure 3 Follow-up CT shows improvement of sigmoid colonic inflammation and disappearance of the pseudodiverticulum.

of free air or pneumoperitoneum. Treatment with broad spectrum parenteral antibiotics was started and continued for 7 d. The patient had an uneventful hospital course; the white blood cell count and C-reactive protein returned to normal within 48 h. Follow-up abdominal CT 4 d later revealed interval improvement with disappearance of the pseudodiverticulum (Figure 3). The patient did well after discharge.

DISCUSSION

About 80%-93% of ingested foreign bodies entering the stomach will be passed through the gastrointestinal tract uneventfully[2]. However, if the ingested foreign bodies are long, narrow, sharp such as toothpicks, the incidence of perforation could be as high as 15%-35%[3]. Patients may manifest symptoms due to bowel wall penetration, peritonitis, or an obstructive process. Toothpick ingestion appears to be commonly implicated in intestinal perforations due to the length and bilateral pointed ends of this foreign body[1]. The most frequent site of injury from ingested toothpicks is duodenum, followed by sigmoid colon[4]. Toothpicks seem to be benign materials from its wooden composition comparing to metallic pin, but surprisingly high mortality rate of toothpick injury (18%) was reported in one study[4]. Moreover, the mortality of patients presenting in shock or with enteric-vascular fistulas was extremely higher (70% and 80%, respectively)[4].

Until recently, toothpick impaction in the lower gastrointestinal tract has been managed by early surgery. One possible explanation for this fact is that most toothpicks are made of wood and may not apparent on imaging studies. Li *et al* reported that toothpicks were apparent on imaging studies only in 14% of the cases[4]. The definite diagnosis was most commonly made at laparotomy (53%), followed by endoscopy (12%)[4]. Another possible explanation is that most patients do not remember swallowing a toothpick. This may lead the physicians to hardly consider toothpick impaction as the cause of patients' symptoms. However, as the skills and devices of endoscopist improve, this approach to management should be reconsidered. Obviously, operative removal of a frankly perforating toothpick is prudent, but it appears that in some cases endoscopic intervention may be accomplished at acceptable rates of morbidity and mortality. In several cases, endoscopic removal of toothpicks injury was described in the literature[1,5-9]. Preceding 5 cases presented with colonic perforation and the last one presented with toothpick impaction in the transverse colon without evidence of perforation. By contrast, severe sigmoid colonic inflammation with pseudodiverticulum formation was noted in our case. Free air or pneumoperitoneum, which is a sign of perforation, was not evident. To our knowledge, colonic pseudodiverticulum associated with toothpick impaction has not been reported. Colonoscopic removal of the foreign body and antibiotics treatment led to an uneventful

recovery.

In summary, we report a case of toothpick impaction with sigmoid colon pseudodiverticulum formation successfully managed with colonoscopy. This case emphasizes the uncommon but possibly mortal hazards associated with the ingestion of toothpicks. Such sharp objects may cause acute inflammation without obvious perforation and present with signs of an acute abdomen. If this unusual but important cause of abdominal pain is taken into account and diagnosed in time, endoscopic management is possible.

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CASE REPORT

Sister Mary Joseph's nodule as the first sign of pregnancy-associated gastric cancer: A case report

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Abstract

Sister Mary Joseph's nodule is an inconspicuous and uncommon clinical sign of advanced malignant disease, especially gastric cancer. Pregnancy-associated gastric cancer is an extremely rare condition and can be difficult to diagnose, due to the absence or misinterpretation of symptoms as pregnancy-related. Diagnostic aids, such as a basic chemistry panel and imaging techniques, may not show any abnormalities. We present a case of a 37-year-old pregnant patient whose umbilical nodule was the first presenting physical sign of gastric cancer, which had metastasized throughout the abdominal and pelvic regions.

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Key words: Sister Mary Joseph's nodule; Gastric cancer; Pregnancy-associated gastric cancer; Umbilical nodule; Metastases in pregnancy

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INTRODUCTION

Sister Mary Joseph's nodule is a rare umbilical lesion most commonly related to advanced malignant abdominal disease, including gastric cancer[1]. Moreover, gastric cancer associated with pregnancy is in itself an extremely rare occurrence. The available information on this subject is scarce and consists mainly of case reports and studies on large patient populations, in which some cases of pregnancy-associated malignancies are included[2-5]. Diagnosis is usually delayed due to non-specific symptoms that are often misinterpreted as pregnancy-associated. The lack of suspicion and general reluctance to perform invasive investigations during pregnancy contribute to late diagnosis. The delay in diagnosis and, consequently, treatment leads to the extremely poor prognosis associated with this disease. We report a case of a 37-year-old woman, whose umbilical metastasis was the first presenting sign of gastric cancer during wk 34 of gestation.

CASE REPORT

A 37-year-old, gravida 3, para 3 woman was admitted during wk 36 of gestation to our obstetrics department for further evaluation. The patient had a history of preprandial, crampy, epigastric pain and diarrhea for several years. Her father died of colorectal cancer in his late fifties and her mother had breast cancer. However, neither Amsterdam II nor Bethesda criteria for hereditary nonpolyposis colorectal cancer (HNPCC) were met by the family[6].

Years previously, the symptoms had been attributed to lactose intolerance. A prescribed diet did not provide any relief, but gastroscopy performed 2 years prior to definitive diagnosis of gastric cancer was normal. Infection with *H pylori* was diagnosed by using the ^{13}C-urea breath test, and its eradication led to amelioration of her symptoms. The complaints recurred less than a year later, with the cardinal symptom of preprandial epigastric pain.

The patient noticed at this time a red, pressure-sensitive lesion with a diameter of 2 cm on her umbilicus. Her general practitioner did not perform any additional investigation of the lesion because he deemed it as harmless. Two months later, her fourth pregnancy was achieved. Melena and intermittent rectal bleeding occurred in wk 10 of gestation, but the symptoms were attributed to pregnancy-related hemorrhoids. Since the umbilical lesion evolved into a nodule with a bumpy irregular surface (Figure 1), she was referred to the department of dermatology. A biopsy was performed at wk 34 of gestation; histological examination revealed a diffuse, undifferentiated, growing carcinoma of unknown origin

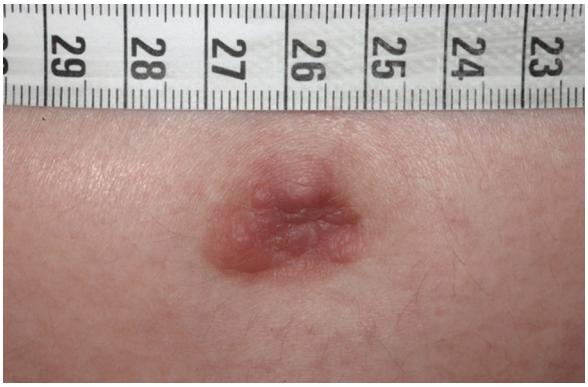


Figure 1 Sister Mary Joseph's nodule.

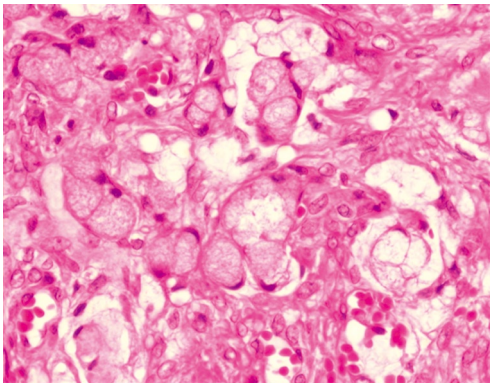


Figure 2 Histology of Sister Mary Joseph's nodule with diffuse, undifferentiated growing carcinoma cells, some containing cytoplasmatic mucin.

(Figure 2), and some cells containing cytoplasmatic mucin. Sister Mary Joseph's nodule was confirmed as the first presenting sign of cancer during pregnancy.

Physical examination upon admission was unremarkable, except for normal signs of pregnancy and pallor. The patient was slightly anemic at the time of presentation, with a hemoglobin level of 7.3 mmol/L and a hematocrit of 34%. Other laboratory data, which included liver function tests and a basic chemistry panel, were within normal limits. Tumor marker assessment revealed elevated serum concentrations for carcinoembryonic antigen (CEA, 293.8 U/mL, normal range < 39.0 U/mL) and cancer antigen-125 (CA-125, 39.8 U/mL, normal range < 35.0 U/mL). Other tumor markers stood within normal range. On ultrasound scan and color duplex sonography, the fetus was developing well with no pathological findings.

The patient was referred to our gastroenterology department where she underwent gastroscopy. A rough ulcer, about 3 cm in diameter, originated from the angular notch. Biopsy revealed a poorly differentiated gastric adenocarcinoma with signet ring cells. Diagnostic imaging (abdominal ultrasonography, thoracic CT and abdominal MRI) showed no clear evidence of metastases. Breast and thyroid sonography showed no abnormalities.

The patient delivered by cesarean section a healthy, 3.2 kg female (Apgar score, 10/10/10). Cesarean section was followed by abdominal and pelvic exploration, which revealed bilateral ovarian tumors (Krukenberg tumors), metastatic spread to the lymph nodes throughout the peritoneal cavity, and tumor infiltration of the spleen and transverse colon. Extensive carcinomatosis of the parietal peritoneum and of the surfaces of the gall bladder and small intestine was present. Total gastrectomy, splenectomy, cholecystectomy, extended right-sided hemicolectomy, bilateral adnexectomy and excision of the umbilicus were performed followed by reconstruction by Roux-en-Y gastrojejunostomy. All the visible tumor tissue was removed. Histological examination showed a poorly differentiated gastric adenocarcinoma with signet ring cells, of mixed type according to Laurén's classification, which was identified in large proportions of the entire stomach, and not only located in the previously described ulcer. The pathologist confirmed stage Ⅲ disease because of peritoneal carcinomatosis, and interpreted the umbilical lesion as metastatic spread and not as continuous skin infiltration by the tumor. The placenta and umbilical cord, however, were free of metastases. Palliative chemotherapy began immediately postpartum. The further postoperative course was complicated by a leak at the blind end of the jejunal loop of the esophagojejunostomy, and a subphrenic abscess with peritonitis. Surgical reintervention was carried out and chemotherapy stopped temporarily. At present, the patient is recovering from surgery and the chemotherapy has been resumed. The baby is doing well.

DISCUSSION

An extended review of literature revealed no previously reported cases of umbilical metastasis as the first presenting sign of gastric cancer during pregnancy.

The term "Sister Mary Joseph's nodule" was coined by Sir Hamilton Bailey in his book, *Physical Signs in Clinical Surgery*, in honor of Sister Mary Joseph, Dr. William Mayo's surgical assistant in the early days of the Mayo Clinic. Sister Mary Joseph identified the relationship between umbilical nodules and advanced intra-abdominal malignancy[7,8]. Sister Mary Joseph's nodule is an umbilical metastasis most commonly found in advanced-stage adenocarcinomas of the upper gastrointestinal tract and ovary. Less commonly, the nodule may be a metastatic lesion from colon or pancreatic cancer[9,10]. The presence of an umbilical metastasis indicates a poor prognosis and is a sign of advanced malignant disease. The survival of these patients has been reported to range from 2 to 11 mo from the time of initial diagnosis[10-13].

Gastric cancer primarily occurs in patients over 50 years of age. The described proportion of young patients with gastric cancer varies from 2% to 15% depending on the age defined as young[14,15]. Female patients have a relative dominance in this group and the incidence of gastric cancer is higher in women over the age of 30 years. The frequency of pregnancy has been increasing in these women and this may lead to a higher coincidence of pregnancy and gastric cancer[2]. Pregnancy-associated gastric cancer is currently attributed to 0.1% of all cases[16]. There is an increased risk of diffuse and scirrhous type carcinomas in this group of patients[4,17,18].

Common presenting signs and symptoms of gastric cancer include dyspepsia, epigastric pain, early satiety,

nausea, vomiting, anorexia, anemia, dysphagia, weight loss and melena. Except for weight loss and melena, all of these symptoms are common in pregnancy. Weight loss can be caused by hyperemesis gravidarum or compensated and masked by the weight gain in pregnancy, and melena may be initially attributed to pregnancy-related hemorrhoids.

In our case, the tumor was advanced at the time of diagnosis. We can only speculate that the delay between the first symptoms and diagnosis might have been several years, possibly due to multiple pregnancies concealing the symptoms during that time. To diagnose diffuse-type gastric cancer is sometimes difficult, if there is no typical lesion and an adequate number of biopsies is taken during gastroscopy. In our case, the cancer was spread throughout the stomach and was not only located in the ulcer region. An adequate number of biopsies taken during previous endoscopies would have made an earlier diagnosis possible. Marconi *et al* have reported a mean delay, from first symptoms to final diagnosis, of 29.3 ± 9.9 wk in young patients without alarm symptoms[19]. The initial diagnosis of gastric carcinoma is often delayed because up to 80% of patients are asymptomatic during the early stages of gastric cancer[20]. When symptoms are present, they can be misinterpreted as simply pregnancy-related. Hormonal stimulation and pregnancy in young women may accelerate the growth of gastric cancer[3,21]. Therefore, we tested for estrogen and progesterone receptors in our patient's tumor and the results were negative. Our patient had consecutive pregnancies with continuous, worsening symptoms. The presence of the umbilical lesion 2 mo before the latest pregnancy strongly suggests that the disease was already at an advanced stage at that time. Gastric cancer should be included, therefore, in the differential diagnosis of unusual or prolonged gastrointestinal symptoms after the first trimester of pregnancy, and in women who have recently been pregnant[15,21]. Coexistent risk factors such as positive family history should trigger immediate further investigation through gastrointestinal endoscopy, which is not contraindicated during pregnancy.

The unique presenting sign of Sister Mary Joseph's nodule indicates an advanced malignant disease with poor prognosis for the mother, but not for the baby. Such nodules might be misinterpreted as eczematous lesions. Based on our case, it is evident that a skin lesion in the umbilical area might indicate malignant disease, and biopsy should be considered if short-term (1-2 wk) local treatment is not successful.

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CASE REPORT

Intestinal pseudo-obstruction: An uncommon condition with heterogeneous etiology and unpredictable outcome

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Abstract

Intestinal pseudo-obstruction (IPO) either acute or chronic is a condition including features of intestinal ileus in absence of mechanical obstruction. Our paper presents such a rare case of idiopathic IPO in a 53-year-old male patient with recurrent episodes of pseudo-obstruction, which were successfully resolved by anticholinesterase agents, motilin agonists or colonic decompression. However, the patient finally underwent total colectomy. Huge colonic dilatation was identified intraoperatively, while histology showed a neuropathic variant of chronic intestinal pseudo-obstruction. Etiologic mechanisms and current therapeutic methods are reviewed in this paper, which concludes that IPO is a condition in which conservative treatment usually fails. Total colectomy with ileoanal pouch may be the only solution in these situations.

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Key words: Intestinal pseudo-obstruction; Anticholinesterase agents; Motilin receptor agonists; Colonoscopic decompression; Total colectomy with ileoanal pouch

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INTRODUCTION

Intestinal pseudo-obstruction is a condition characterized by intestinal obstruction in the absence of any mechanical obstructing lesion. Pseudo-obstruction can exist in the small bowel or in the colon and can be either acute or chronic. Acute colonic pseudo-obstruction (ACPO), also known as Ogilvie syndrome, is the most common form of intestinal obstruction. It occurs commonly in postoperative period of surgical patients, or as a response to a nonsurgical acute illness. Chronic intestinal pseudo-obstruction (CIP) is a rare and heterogeneous clinical syndrome, with a relapsing and remitting pattern that appears either in idiopathic (as consequence of a primary abnormality of the gut, namely a visceral myopathy or neuropathy) or in other non-intestinal disorders.

Our paper presents a rare case of idiopathic CIP in a 53-year-old Caucasian male showing the neuropathic variant with hypoplasia of ganglionic cells of the rectum, sigmoid and descending colon.

CASE REPORT

The patient complained of abdominal distension, vomiting and no bowel movements for 5-7 d. He had this history three or four years ago, with episodes of progressive abdominal distension and constipation, and was diagnosed as irritable bowel disease. In the last year, these episodes increased in severity and frequency, leading to food abstention and secondary denutrition.

The episodes of bowel movements were more and more severe. However, as mechanical obstruction was systematically ruled out, the patient was discharged after certain symptomatic treatment each time. Finally, after four or five such episodes of "unexplained" pseudo-occlusion, he was admitted to the gastroenterology unit for further investigation.

Physical examination during these obstructive episodes revealed huge abdominal distension with hypoactive bowel, but no abdominal mass or peritoneal signs. The patient had no history of metabolic, neurological, cardiovascular or pulmonary disease, abdominal or pelvic operations, abdominal cancer, intra-abdominal inflammation or abdominal trauma. He did not take other concomitant medication.

Biologic screening showed normal serum electrolyte concentration, hemoglobin, hematocrit, white blood cells, blood glucose, creatinine, and coagulation profile. There was no evidence of metabolic diseases like diabetes, porphyria, amyloidosis. Pulmonary imaging was normal. Abdominal ultrasound did not find any sign of small bowel or colonic mechanical obstruction. Distended



Figure 1 Supine radiograph with no preparation showing massive colonic distention especially the transverse colon, but without air-fluid levels.



Figure 3 Laparotomy showing massive distension of the whole colon with no perforation or volvulus. Total colectomy removed a completely atonic colon which was 2.5 m in length and 25 cm in diameter. Ileoanal pouch was made subsequently.

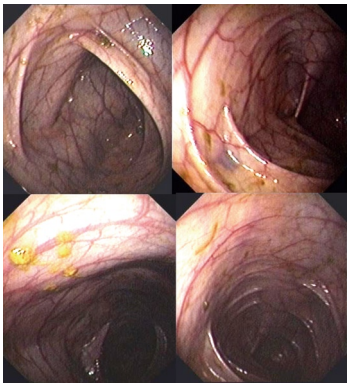


Figure 2 Colonoscopy showing huge colonic distension involving all segments, with a diameter of the rectosigmoid and descending colon greater than 12 cm, and that of the ascending colon and cecum greater than 16-18 cm.

bowel segments were observed, but free peritoneal fluid, pendulating peristalsis, bowel wall edema, or fixed masses of aperistaltic, fluid-filled distended intestinal loops were not observed. Radiographs showed signs of marked colonic dilatation (Figure 1) especially in the cecum and ascending colon, but no air-fluid levels were found in the colon or in the small intestine. Abdominal CT-scan showed nothing apart of bowel distension and aerobilia while the pulmonary and cranial scans were normal.

Colonoscopy showed incredible colonic distension (Figure 2) up to the cecum and decompressed the abdomen. No obstructive, diverticular or mucosal changes were noticed. Randomized multiple mucosal biopsies were also normal. CIP syndrome was strongly suspected, which was enforced by the fact that after intravenous administration of neostigmine (2 mg) rapid evacuation of flatus and stool and significant decrease in abdominal distention were observed.

The patient was discharged and oral neostigmine was administered regularly (15 mg per day). Two weeks after apparent amelioration of bowel habits, the patient had a new pseudo-obstructive accident, which remitted again with colonoscopic decompression and intravenous neostigmine. Oral erythromycin was introduced at discharge, at a dose of 500 mg, three times per day, resulting in fewer episodes of constipation, pain and vomiting and more regular bowel actions, which increased from 1.5 per week to 3 per week for a period of three months. Unfortunately, after this period, recurrence occurred and because of failure in all other conservative therapies (including prokinetics, octreotide, propranolol) the patient finally underwent colectomy.

Intra-operation examination revealed massive colonic distension, the large bowel length was more than 2.6 meters and the cecum diameter was larger than 15 cm (Figure 3). After a total colectomy with ileoanal pouch, the condition was resolved satisfactorily. Full thickness colonic sections were histologically analyzed. No myopathic abnormality was found but ganglionic cells were absent in the last 7 cm of the rectum, and marked depletion of neural plexuses with hypertrophy of the external muscular layer was identified in sigmoid and descending colon (Figure 4), thus diagnosis of neuropathic CIP was established.

No postoperative complication and obstructive episode occurred after colectomy. Prokinetics were discontinued, and the weight of the patient became normal. Apart from some diarrheal stools, which occurred in the first two months after operation, the bowel movements did not exceed 7.5/wk, with no need for antidiarrheal drugs or nutrient supplementation.

DISSCUSION

Megacolon is a descriptive term, without etiologic and pathophysiologic implication. It can be acute or chronic, each category being subdivided in idiopathic and secondary to various disorders. Idiopathic forms are usually assigned to variants that lack decisive elements for diagnosis on routine stains, although specialized immunohistochemical techniques frequently reveal unrecognized disorders, such as primary biochemical disorders of the gut muscle[1]. Toxic megacolon is classically not included in these categories.

When colonic obstruction is acute[2] and associated with a recent, major organic disorder, trauma, or surgical procedure as shown in Table 1, the term of Ogilvie's syndrome is commonly used. Imbalance in the autonomic nervous system is the presumed physiopathologic factor postulated by Sir Heneage Ogilvie when he firstly described ACPO in 1948[3]. In his original work, Ogilvie considered that high parasympathetic tone combined with low sympathetic activity of the colon leads to regional contraction and thus, functional obstruction, but little direct evidence supports this theory. Conversely, many recent observations suggest that parasympathetic deprivation combined with high sympathetic tones is the main factor contributing to pseudoobstruction[4,5]. The

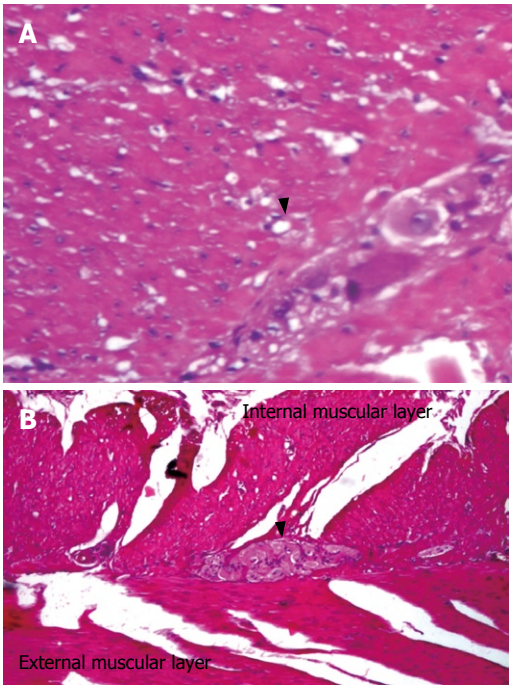


Figure 4 Full-thickness serial biopsies stained with haematoxylin-eosin (× 100) from the removed colon showing no ganglionic cells in the last 7 cm of the rectum. Marked depletion of ganglionic cells (arrow) and hypertrophy of the external muscular layer were identified in sigmoid (**A**) and descending colon (**B**).

efficacy of cholinesterase inhibitors in reversing ACPO[6,7], as well as observations regarding colonic atony similar to Hirschsprung disease after interruption of the sacral parasympathetic nerves suggest that a high sympathetic and/or low parasympathetic activity[8] is more likely to induce ACPO. Some other factors may also contribute, like increase in nitric oxide production[9] or inhibition of motilin receptors to ACPO[10].

Chronic intestinal pseudo-obstruction can be congenital or acquired, both forms can be subdivided into neuropathic and myopathic variants (Table 2).

The most common form of *neuropathic CIP* is Hirschsprung disease, caused by a congenital absence of intramural plexuses that mediate relaxation (aganglionosis), leading to narrowing of a segment of large intestine that impairs progression of colonic content and secondarily generates upstream dilatation. Aganglionosis results from arrest of caudal migration of embryonic cells that will form the myenteric plexus from the neural crest in early embryogenesis, and, consequently this disease is included in a group of disorders called neurocristopathies [11]. The aganglionic segment usually does not exceed 5-15 cm in length, being located in the distal rectum, but in certain cases, it can be longer, extending to the sigmoid or even may exceptionally extend to the entire colon.

Variants of Hirschsprung disease include type Ⅱ (b) multiple endocrine adenomatosis[12] and some other conditions as hypoganglionosis and neuronal intestinal dysplasia. In type Ⅱ multiple endocrine neoplasia (MEN Ⅱ) and Hirschsprung's disease, a mutation of an autosomal dominant gene localized to a 250-kilobase interval containing the RET proto-oncogene is mapped to chromosome 10q11.2[13,14]. The mutation enhances the function of a tyrosine-kinase receptor in MEN Ⅱ, while in Hirschsprung disease it inactivates the protein. Apart from the four variants of this mutation that damages the structure of the tyrosine kinase domain of the RET protein, other mutations have been also recently described[15] in Hirschsprung's disease. In animal models,

Table 1 Major causes for acute intestinal pseudo-obstruction (adapted from 2)

Post-surgery	Abdominal, orthopedic, neurologic, urologic, cardiac interventions, caesarean section, gynecological/pelvic surgery, renal transplantation
Pulmonary diseases	Pneumonia, mechanical ventilation, adult respiratory distress syndrome
Electrolyte disturbances and metabolic disorders	Hyponatremia, hypokalemia, hypocalcaemia or hypocalcaemia, hypomagnesaemia, alcohol abuse, liver and renal failure
Cardiovascular diseases	Myocardial infarction, cerebrovascular accident, congestive heart failure
Malignancy	Retroperitoneal neoplasia, leukaemia, metastatic cancer, pelvic radiotherapy
Infective/inflammatory	Acute cholecystitis, pancreatitis, peritonitis, systemic sepsis, herpes zoster infection, gram-negative infections,
Neurologic	Parkinson's disease, Alzheimer, multiple sclerosis, low spinal cord disease
Traumatic	Pelvic trauma, spinal cord injuries, femoral fracture
Medications	Narcotics, opiates, anticholinergics, clonidine, amphetamines, phenothiazines, steroids

Table 2 Major causes for chronic intestinal pseudo-obstruction (adapted from 11)

	Neuropathic	Myopathic
Idiopathic	-Hirschsprung disease	-Nonfamilial hollow visceral myopathy
Acquired	-Familial visceral neuropathies -von Recklinghausen disease -Infiltrative diseases (amyloidosis) -Metabolic, endocrine and electrolyte diseorders (porphyrias, diabetes mellitus, hypothyroidism, hypokalemia) -Neurologic diseases (brainstem tumors, multiple sclerosis, dysautonomias) -Infectious diseases (trypanosomiasis, cytomegalovirus infections) -Neoplastic diseases (bronchial small cell carcinoma, carcinoids, pheochromocytoma) -Drugs (narcotics, antihypertensives, antidepressants)	-Familial visceral myopathies -Infiltrative diseases (amyloidosis, systemic sclerosis) -Myotonic and other dystrophies

Parisi *et al*[16] investigated the regulation of Hox11L1, a gene expressed by enteric neurons in Hox11L1- transgenic null mice and concluded that expression of the transgene and penetrance of pseudo-obstruction are influenced by one or more modifier genes depending on the genetic background, some of them having a putative role in human disease.

Congenital aganglionosis is associated with other genetic disorders like Down's and Laurence Moon Bardet Biedel syndromes. Type IV Waardenburg-Shah syndrome (WS4) is characterized by association of Waardenburg features (WS, depigmentation and deafness) and absence of enteric ganglia in the distal part of the intestine. Mutations in the genes EDN3, EDNRB and SOX10 have been reported in patients presenting either the classical form of WS4, or chronic intestinal pseudo-obstruction and WS features[17].

Among acquired conditions that generate CIP, Chagas disease is widely known. In this disease, the neurotoxin generated by Trypanosoma cruzii has a deleterious effect on the enteric nervous system. Other infections, like cytomegalovirus can have similar consequences by unknown mechanisms. Metabolic disorders like diabetes mellitus and porphyria, as well as infiltrative diseases (systemic sclerosis, amyloidosis) can also lead to neuropathic variants of CIP. Some drugs (antidepressants, anticholinergics, vincristine) can interfere with myenteric plexus activity. Mechanisms of neuronal damage may be toxic or immune, as in patients treated with buserelin[18], an analogue of gonadotropin-releasing hormone (GnRH) in which formation of buserelin-induced anti-GnRH antibodies can destroy GnRH-producing neurons of the myenteric plexus.

Little is known about the mechanisms generating pseudo-obstruction in neuropathic forms of CIP, either idiopathic or acquired. It seems that lack of normal pacemaker activity, usually generated by the interstitial cells of Cajal (ICC), could account for obstruction[19]. ICC, normally located between the myenteric plexus ganglia, can be stained immunohistochemically with CD117 as well as with CD34, which also stains a population of fibroblasts intimately associated with ICC. In some CIP patients, both CD117 and CD34 are absent or severely reduced, while in controls ICC staining is normal, and moreover, the CD34-positive fibroblasts associated with ICC are absent in these cases[20].

In aganglionosis, the aganglionic segment still exhibits spontaneous phasic contractions that contribute to pseudoobstruction. The mechanism that generates these myogenic contractions and the generation of Ca(2+) waves that underlie contractions of the longitudinal muscle (LM) and circular muscle (CM) was recently studied by Spencer *et al*[21] in a mouse model of Hirschsprung's disease. In control mice, during the intervals between colonic migrating motor complexes (CMMCs), Ca(2+) waves discharged asynchronously between the LM and CM, while a burst of discreet Ca(2+) waves firing simultaneously was noticed in both muscle layers, with an increased propagation velocity of Ca(2+) waves. In aganglionic mice, CMMCs were absent and Ca(2+) waves between the two muscle layers fired asynchronously, despite an increased propagation velocity, resulting in a sporadic and sustained asynchrony in Ca(2+) waves firing between the LM and CM.

Neurotransmitters like nitric oxide (NO) can also play a role in generating pseudo-obstruction. NO is synthesized by the activation of neuronal NO synthase (nNOS) in the myenteric plexus in response to nerve stimulation and determines relaxation of the smooth muscle. Reduction in nNOS expression, associated with impaired local production of NO, may be responsible for some motility disorders including Hirschsprung's and Chagas diseases[9] where it is conceivable that extrinsic denervation may up regulate nNOS expression, resulting in enhanced muscular relaxation and disturbed peristalsis.

Myopathic variants of CIP have been intensively studied, although there are a lot of controversies regarding this inhomogeneous group. Visceral myopathy is a form of CIP characterized by vacuolar degeneration, atrophy and fibrosis of the intestinal muscle layer without inflammatory cells[22]. The familial form occurs in about 30% of cases having a transmission mostly autosomal recessive.

Mitochondrial DNA defects, as well as mitochondrial beta-oxidation disorders can also determine intestinal pseudo-obstruction associated with or not with progressive skeletal myopathy and neuropathy in some cases. Defects in mitochondrial proteins like mitochondrial trifunctional protein[23], an enzyme complex catalyzing the last three steps in the beta-oxidation cycle, causes intestinal pseudo-obstruction by activation of extramitochondrial fatty acid oxidation pathways with generation of excessive reactive oxygen species, leading to visceral myopathy. The muscle biopsies in such cases show intestinal smooth myocytes with bulbous protrusions filled with lateralized mitochondria, while histochemistry can show a decreased reactivity of cytochrome-c oxidase and marked reductions in other mitochondrial enzyme activities like NADH: Q1 and NADH: O2 oxidoreductases. Although considered as causative factors for myopathic CIP, mitochondrial disorders can also determine neuropathic pseudo-obstructions, or at least, can be associated with other neuronal intestinal malformations[24].

Deficiencies of alpha smooth muscle actin (ASMA) expression in intestinal smooth muscle are associated with CIP. Knowles *et al*[25] found that 24% of CIP patients have absent or partial ASMA immunostaining in the circular muscle layer, while smooth muscle alpha-actin staining is preserved in the longitudinal muscle and controls. Other authors[26] also tested the hypothesis that deficient ASMA expression in intestinal smooth muscle is specifically associated with clinical evidence of CIP using primary antibodies to ASMA, smooth muscle myosin heavy chain and desmin. While the staining for the later two antibodies was similar in patients and controls, ASMA expression was absent or minimal in all CIP patients but also in a great number of controls, leading to the conclusion that there is not enough evidence to confirm this theory. In contrast, Silk and his group[27] found that 58% of CIP patients have complete or partial deficiency of alpha-actin epitope staining in the inner circular layer of small intestinal smooth muscle, considering this deficiency as a biomarker of disease rather than a cause of CIP.

Immune disorders can seldom determine CIP. Intestinal pseudo-obstruction has been described in less than 20 cases in association with systemic lupus erythematosus[28] and, in extremely rare situations, autoimmune enteric leiomyositis[29] can determine pseudo-obstruction. It seems that coexisting disorders like autoimmune hepatitis can favor expansion of autoreactive T cells to homologous self-antigens in the muscularis propria, with smooth muscle fibers degenerated but with the myenteric neuronal plexus preserved.

Treatment of CIP is not standardized. Propulsive laxatives, prokinetics, anticholinesterase agents, motilin receptor agonists, and even octreotide, clonidine and propranolol have been tried in small series, but given the rarity of this condition, few controlled trials have been conducted. A number of reports have evaluated the efficacy of a prokinetic agent, cisapride, in the treatment of chronic intestinal pseudo-obstruction[30,31], showing some evidence for short-term improvements, but because of cardiac toxicity, this agent is actually restricted. It has been demonstrated that intravenous administration of neostigmine, 2.5 mg over 2 to 3 min, leads to prompt resolution of acute colonic pseudo-obstruction[32,33] by inhibiting the destruction of acetylcholine by acetylcholinesterase, which facilitates transmission of impulses across the myoneural junction. However, in CIP the results are not so obvious, and the role of neostigmin in treating these patients has not been formally evaluated, except in sporadic case reports[34,35]. Some papers[10] report improvement in gut function with use of erythromycin, given orally or intravenously in a small number of patients with CIP, suggesting that it can be of benefit for preventing and attenuating the episodes of pseudo-obstruction. Erythromycin inhibits the binding of motilin to the receptors on gastrointestinal smooth muscle, and, despite the relative absence of motilin receptors in the lower gut, there is evidence that supports its role in accelerating colonic transit in patients with CIP[36,37].

Colonoscopic decompression[38-40], with or without placement of a decompression tube, is accepted as a standard method to remove air from the colon in CIP, allowing decompression and reducing the risk of perforation. Although this procedure may be hazardous and sometimes difficult to perform because of poor preparation, it is usually efficient and passage of the endoscope to the hepatic flexure always permits decompression of the cecum in most patients. Unfortunately, dilatation usually recurs following decompression.

Surgical treatment is usually reserved to refractory situations, on a case-by-case basis. The options include total abdominal colectomy with ileorectal anastomosis, total proctocolectomy with ileostomy, or with ileoanal anastomosis[41-43] depending on the site of the colon affected.

In conclusion, CIP is a rare disease in which colonic dilatation can be impressive. Its etiology is not obvious, since multiple neuropathic and myopathic abnormalities can account for it. Although conservative treatment with anticholinesterase agents, motilin agonists or colonic decompression is recommended, its long-term efficacy is doubtful and colectomy is sometimes needed.

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CASE REPORT

Aggressive behaviour of solid-pseudopapillary tumor of the pancreas in adults: A case report and review of the literature

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Abstract

Solid-pseudopapillary tumor (SPT) is a rare neoplasm of the pancreas that usually occurs in young females. It is generally considered a low-grade malignant tumor that can remain asymptomatic for several years. The occurrence of infiltrating varieties of SPT is around 10%-15%. Between 1986 and 2006, 282 cystic tumors of the pancreas were observed. Among them a SPT was diagnosed in 8 patients (2.8%) with only one infiltrating variety. This was diagnosed in a 49-year-old female 13 years after the sonographic evidence of a small pancreatic cystic lesion interpreted as a pseudocyst. The tumor invaded a long segment of the portal-mesenteric vein confluence, and was removed with a total pancreatectomy, resection of the portal vein and reconstruction with the internal jugular vein. Histological examination confirmed the R-0 resection of the primary SPT, although a vascular invasion was demonstrated. The postoperative course was uneventful, but 32 mo after surgery the patient experienced diffuse liver metastases. Chemotherapy with different drugs was started. The patient is alive and symptom-free, with stable disease, 75 mo after surgery. Twenty-five patients with invasion of the portal vein and/or of mesenteric vessels were retrieved from the literature, 16 recent patients with tumor relapse after potentially curative resection were also retrieved. The best treatment remains a radical resection whenever possible, even in locally advanced or metastatic disease. The role of chemotherapy, and/or radiotherapy, is still to be defined.

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Key words: Solid-pseudopapillary tumor; Pancreatectomy; Vascular resection; Liver metastases; Follow-up

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INTRODUCTION

Solid pseudopapillary tumor (SPT) of the pancreas was first reported by Frantz in 1959[1], and represents a rare tumor that occurs most frequently in young women (90%). The widespread use of imaging techniques, together with a better knowledge of the disease, has prompted a rapidly increasing number of case reports. A recent review of the English literature from 1933 to 2003 collected a total of 718 SPTs, including pediatric cases[2].

Although SPT is considered an indolent lesion with a low malignant potential and a favorable prognosis after surgical resection, some cases of locally infiltrating and metastatic variety, or recurrences after surgery, have been reported. Moreover, long-term survival is described even for metastatic disease, suggesting a widely variable and not clearly elucidated biology of this tumor.

We report a patient with an infiltrating SPT that underwent total pancreatectomy and wide vascular resection. The tumor recurred in the liver 32 mo after surgery and chemotherapy was started. Between 1986 and 2006, we observed 8 SPTs among 282 cystic tumors of the pancreas (2.8%), the remaining 7 patients were cured by surgery and are alive and disease-free (Table 1).

A literature review of infiltrating SPTs allowed us to find 25 patients with invasion of the portal vein and/or of mesenteric vessels, and 16 recent patients with tumor relapse after potentially curative resection.

CASE REPORT

A 49-year-old woman was admitted to another hospital

Table 1 Patient characteristics

Patient	Sex	Age	Site	Size	Treatment	Follow-up (mo)
1	F	54	Tail	4	DP	A, NED (252)
2	F	13	Body	4	CP	A, NED (171)
3	F	32	Tail	7	DP	A, NED (120)
4	F	31	Tail	14	DP	A, NED (108)
5	F	49	Body-tail	10	TP	A, WD (77)
6	F	20	Tail	10	DP	A, NED (75)
7	F	14	Tail	10	DP	A, NED (48)
8	M	59	Tail	11	DP	A, NED (12)

DP: Distal pancreatectomy; CP: Central pancreatectomy; TP: Total pancreatectomy; A: Alive; NED: No evidence of disease; WD: With disease.

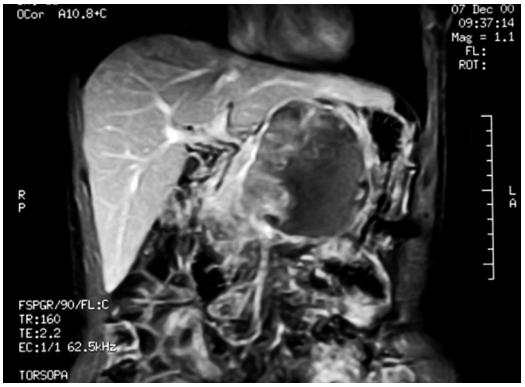


Figure 1 MRI of the abdomen showing a large, solid-cistyc mass of the pancreas.



Figure 2 CT of abdomen showing multiple liver metastase with solid-cystic appearance.

in February 2001 because of an 8 mo history of palpable abdominal mass. A 2-cm cystic lesion of the tail of the pancreas was detected by an abdominal ultrasonography 13 years ago, and interpreted as a pancreatic pseudocyst. Physical examination revealed a hard mass in the left upper quadrant of the abdomen. Routine laboratory tests including carcinoembryonic antigen (CEA) and CA 19-9 were within the normal range. A CT-scan showed a 10-cm abdominal mass with an irregular low density pattern in the body and tail of the pancreas. At laparotomy a neoplastic mass involving the body and tail of the pancreas, with encasement of the portal and superior mesenteric vein confluence, was found. A core biopsy of the mass was performed, but it was referred as non diagnostic. The tumor was judged unresectable. Soon after the patient was referred to our department, MRI showed a mass of about 8 cm in diameter with an irregular intensity both in T1 and in T2 enhanced-images, surrounded by a capsule (Figure 1). The splenic vessels were encased by the tumor, but the portal vein, superior mesenteric vessels, and celiac trunk were apparently free. A positron emission tomography with 18-fluorodeoxyglucose (^{18}FDG-PET) showed a peripheral, pathologic uptake with central absence of metabolism (SUV = 3.5). In the presumption of a cystadenocarcinoma of the pancreas the patient underwent explorative laparotomy. A thick-walled encapsulated mass, 10 cm × 10 cm in size, was found in the body and tail of the pancreas. There were no metastatic lesions in the liver or distant organs, but the portal-mesenteric vein confluence was infiltrated by the tumor. A pylorus-preserving total pancreatectomy with resection of the portal vein was performed. The mesenteric-portal vein was reconstructed with the interposition of the right internal jugular vein.

The postoperative course was uneventful and no adjuvant therapy was given. The patient was discharged from the hospital 16 d after surgery.

Histopathology

The pathological investigation showed a 10.0 cm × 10.0 cm × 9.5 cm pancreatic tumor partially demarcated by a fibrous capsule. Gross pathomorphology of the cut surface showed an inhomogeneous solid appearance with areas of extensive necrosis. Although the mass was partially encapsulated, it directly invaded the peripancreatic fat tissue and the muscular layer of the portal vein with no involvement of the resection margins. Microscopically, solid areas composed of monomorphic epithelioid cells showing venous invasion and a moderate cellular atypia without mitotic activity were noted. Characteristic pseudopapillary formations were frequently observed. Twenty-seven lymph nodes examined were free of tumor. Immunohistochemistry was uniformly positive for vimentin, alfa-1-antitrypsin and CD10 staining. Strong immunoreactivity was also found for neuron-specific enolase (NSE). Focal reactivity was detected for synaptophysin, while negative reactions were found for cytokeratins (pan-cytokeratin, CAM 5.2), CEA, progesterone and estrogen receptors.

On the basis of these characteristic morphologic and immunohistochemical findings, the diagnosis of locally invasive SPT of the pancreas was made.

Follow-up

Follow-up included clinical examination, routine laboratory tests, abdominal US and/or CT/MRI every 6 mo. Thirty-two months after surgery, abdominal CT showed multiple metastatic lesions throughout the entire liver (Figure 2). The greater lesions (from 1.0 cm to 2.5 cm) were present in segments Ⅳ, Ⅵ, Ⅶ, and Ⅷ of the liver. The patient was asymptomatic. A whole body ^{18}FDG-PET confirmed some areas of pathological metabolic activity in the liver. A percutaneous liver biopsy under US guidance was performed. The histology showed a metastatic SPT of the

Table 2 Treatment and outcome of patients with SPT involving vascular structures

Author	Yr	Sex	Site of infiltration	Therapy	Follow-up (mo)
Sanfey *et al*[7]a	1982	F	SMV	TP, vascular resection and reconstruction	7, A&W
Dales *et al*[8]a	1983	F	PV	TP, vascular resection and reconstruction, RT	A&Wd
Fried *et al*[9]a	1985	F	PV and HA	Unresectable, RT	36, A&W
Matsuda *et al*[10]a	1987	F	PV and liver metastasis	Unresectable, Chemoembolization, CT	60, AWD
Matsunou & Konishi[11]a	1990	F	PV and peritoneal dissemination	Unresectable, RT & CT	47, DOD
Zinner *et al*[12]a	1990	F	PV	DP, vascular resection and reconstruction	48, A&W
	1990	F	PV	TP, vascular resection and reconstruction	84, A&W
Tsunoda *et al*[13]a	1991	M	PV	Whipple, vascular resection and reconstruction	22, A&W
Nishihara *et al*[14]a	1993	F	Venous invasion	Resected[b]	3, A&W
		F	Venous invasion	Resected[b]	13, A&W
		F	PV	Whipple, vascular resection and reconstruction	66, DUC
Jeng *et al*[15]a	1993	F	PV and SMA	Unresectable, Roux-en-Y cystojejunostomy	83, DUC
		F	PV	Whipple, vascular resection and reconstruction	40, A&W
Saiura *et al*[16]	2000	F	PV with thrombus, liver metastases	DP with splenectomy, extended right lobectomy, partial resection of the liver, vascular resection	8, A&W
Martin *et al*[17]	2002	NA	SMA encasement	Unresectable	156, alive
		NA	SMA encasement	Unresectable	12, alive
Goh *et al*[18]c	2003	F	PV	PD, vascular resection and reconstruction	67, NED
Podevin J[20]	2003	F	SMV	PD, vascular resection and reconstruction, segmental colectomy	12, A&W
Usatoff *et al*[21]	2004	F	SMV	PD, vascular resection and reconstruction	5, NED
Hassan *et al*[22]	2005	F	PV, colon	PD, vascular resection and reconstruction. Enucleation of liver metastases after 12 mo	18, NED
Chen *et al*[23]	2005	F	PV, liver metastasis.	Resection of both primary and metastatic lesions and the portal vein	36, A&W
Frago *et al*[24]	2006	F	PV	Duodenum preserving subtotal pancreatectomy, vascular resection and reconstruction	76, A&W
Hao *et al*[25]	2006	F	SMA, SMV	Unresectable, internal drainage	8, NED
Zauls *et al*[26]	2006	F	PV and SMV	Unresectable, RT	18, alive
Tipton *et al*[27]	2006	NA	SMV	Unresectable, gastroenteroanastomosis	92, DOD
Cheng-Hong *et al*[28]	2006	F	PV	PD, vascular resection and reconstruction	> 12, NA
		F	SMV	PD, vascular resection and reconstruction	> 12, NA
Present case	2007	F	SMV	TP, vascular resection and reconstruction, CT	77, AWD

[a]Patients included in Mao's review[3]; [b]Patients erroneously included as vascular invasion, histological venous invasion was only present; [c]Previously reported by NG *et al*[18]; [d]Duration of follow-up not stated; SMA: Superior mesenteric artery; HA: Hepatic artery; PV: Portal vein; SMV: Superior mesenteric vein; RT: Radiotherapy; CT: Chemotherapy; PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy; TP: Total pancreatectomy; NED: No evidence of disease; NA: Not available; DUC: Died of unrelated cause; AWD= Alive with disease.

pancreas. Immunoreactivity for estrogen and progesterone receptors was negative.

Radical excision of metastatic nodules was considered unfeasible, and chemotherapy was started. The patient received 6 cycles of weekly gemcitabine (1000 mg/m^2) and tolerated it quite well. Afterwards, different regimens of drugs were tested because of slow disease progression (size and number of liver metastases, lymph node metastases): 3 cycles of gemcitabine + irinotecan, 8 cycles of oxaliplatin + irinotecan + capecitabine, 6 cycles of gemcitabine + capecitabine, 2 mo of weekly 5-FU. Finally, from September 2006 until present, the patient received capecitabine (2000 mg/m^2). CT-scan showed stable disease, and the patient was alive and symptom free at the time we wrote this paper.

DISCUSSION

SPT is a rare neoplasm of the pancreas, accounting for only 1%-2% of all exocrine pancreatic tumors[3]. We observed 8 SPTs among a total of 282 (2.8%) cystic tumors of the pancreas from 1986 to 2006 (Table 1). SPT is generally considered a tumor with a low malignant potential, usually occurring in young females in their second or third decade of life[2,4]. Although resection of the tumor provides a 5-year survival rate of 97%, local recurrence or distant metastases can occur in a significant number of patients[4]. Moreover, in a minority of patients a locally advanced or metastatic disease is discovered at their initial presentation, as in our 49-year-old patient. However, when she was 36-year-old, a 2-cm cystic mass was incidentally discovered in the tail of the pancreas and misinterpreted as a pancreatic pseudocyst and no further investigation was made until the palpable abdominal mass was discovered. Therefore it is reasonable to believe that a SPT was present at least 13 years before the mass was discovered. The doubling time, based on the Schwartz' formula[5], was 677, a value between 240 and 765 d of the two other patients in whom the doubling time was reported[6]. This finding, together with the absence of mitotic activity, confirms the slow growing pattern of this neoplasm, but also the need of a regular follow-up for pancreatic cystic lesions left unresected.

At presentation, the tumor showed an infiltrative pattern invading a segment of the portal vein that was *en-bloc* resected with the entire gland in order to achieve tumor-free margins. Locally infiltrative SPTs of the pancreas occur infrequently. A recent review of the English literature until December 2003[2] showed that among 497 patients with data on invasion or metastases,

Table 3 Patients with recurrent disease after potentially curative resection

Author	Yr	Extrapancreatic disease at diagnosis	Therapy	Site, time of recurrence (mo), therapy	Mo from 1st recurrence & follow-up
Levy *et al*[29]	1997	Peritoneal metastases	DP, Omentectomy, lymphadenectomy	Peritoneal carcinomatosis (8): complete resection. Liver (16)	29, AWD
Shimizu *et al*[30]	1999	No	DP	Liver (17)	3, DOP
Rebhandl *et al*[31]	2001	No	SPDP	Peritoneum-omentum (24) radical resection.	> 54, NED
Martin *et al*[17]	2002			Spleen, peritoneum (36) tumor debulking; CT. Recurrence lesser omentum (>72) tumor resection, interferon.	
		Liver	Resection	Liver, NA	8, DOD
		Liver	Resection	Liver, NA	72, AWD
Gedaly *et al*[32]	2002	no	DCP	Liver (36), hepatectomy; liver (72), RF	48, AWD
Tang *et al*[33]	2005	No	DCP	Liver, (3)	13, DOD
Takahashi *et al*[34]	2005	Liver (multiple nodules)	DP, adrenalectomy & enucleation of 38 hepatic nodules	Liver (14, 27, 38)/enucleation of 14, 21, 19 nodules; lung (41)/ lung resection	46, AWD
Huang H-L *et al*[35]	2005	no	SPDP	Liver (168)	10, DOD
Kang *et al*[36]	2006	Peripancreatic nodes	DP	Liver, CT, TACE,(9), Surgery + RF	17, DOD
Tipton *et al*[27]	2006	Incomplete resection	DP	Subcutaneous (14): removed; intra-abdominal recurrences removed (?)	94, NED
Cao *et al*[37]	2006				
		no	UR	Liver, (57)	3, DOD
		no	UR	Liver, (72)	0, AWD
		no	UR	Liver (11), resection	110, NED
Adamthwaite *et al*[38]	2006	nodes	DCP	Liver (2)	0.5, DOD
Nagri *et al*[39]	2007	duodenum	Resection	Liver (48)	48, AWD
Present patient	2007	No	Resection	Liver (32), CT	45, AWD

SPDP: Spleen preserving distal pancreatectomy; DP: Distal pancreatectomy; PD: Pancreaticoduodenectomy; UR: Unspecified resection; TACE: Transarterial chemoembolization; CT: Chemotherapy; RF: Radiofrequency; NA: Not available; DOD: Died of disease; DOP: Died of procedure (TAE); AWD: Alive with disease; NED: No evidence of disease.

portal vein was involved in 26 patients (5%), other organs (spleen, colon, duodenum, *etc*.) in 44 (9%), but the details on treatment and outcome of these patients were not reported. We retrieved 13 patients[7-15] previously reported as "invasion of portal and/or mesenteric vein" by Mao *et al*[3], two of them[14] were actually "histological venous invasion" and were then excluded from further consideration. Fourteen patients reported after 1995 were also retrieved[16-28] for a total of 26 patients including our patient, with SPT involving vascular structures (Table 2). Liver metastases were also present in three patients[10,16,23], invasion of colon[22] or peritoneal dissemination[11] in one each. Seventeen patients[7,8,12,13,15,16,19-24,28] underwent vascular resection and reconstruction without operative mortality. Liver metastases were also removed in two patients together with the primary tumor[16,23]. One died of unrelated disease[15] and all the others are still alive: one after enucleation of liver metastasis 18 mo after pancreatic resection, one (our patient) with liver metastases. The mean and median survival periods of time were 36.9 and 36 mo (range 5-84). Nine patients[9-11,15,17,25-27] were unresectable because of vascular infiltration, liver metastases or peritoneal dissemination. The mean and median survival periods of time were 55.5 and 47 mo (range 8-156). Two of them died of disease after 47 and 99 mo, one died of unrelated cause after 71 mo and one is alive with disease after 60 mo. Based on these data, vascular resection seems not so useful, but we must consider a publication bias due to the usual earlier publication of the results of resective surgery when compared to palliative and explorative surgery or to no surgery at all. Unfortunately, the long term results of many patients who underwent vascular resection have not been reported.

Recurrence after apparently radical resection of a SPT can occur in 10%-15% of cases, and the liver is the most common site[2-4,16,29] (Table 3). Resection of liver metastases is possible if liver involvement is limited[16,23,32,34,36,37]. In our patient, the tumor recurred diffusely in the liver 32 mo after a R-0 resection. Unfortunately, radical excision of liver metastases was not possible, and the patient received chemotherapy with a substantially stable disease 77 mo after initial diagnosis and 45 mo after tumor relapse. The long term survival after appearance of liver metastases has been reported in some patients after surgical treatment[32,34,37], and even without radical treatment[17,39], because of the indolent nature of the disease. However, other patients died within 2 years from appearance of liver metastases[17,30,33,35-38]. Also the appearance of hepatic metastases varies greatly from patient to patient, from a minimum of 2[38] to a maximum of 168[35] mo. At present, there are no established clinical or histological criteria to predict the biological behavior of SPT. While invasion of blood vessels, perineural infiltration, invasion of adjacent structures, high degree of cellular polimorfism, and elevated mitotic rate are suggested to be associated with metastases and recurrence[3,11,14,20,27,33]. Absence of these features does not preclude malignant behavior[30,33,38]. Our patient showed only venous invasion and a moderate

cellular atypia without mitotic activity. In a retrospective study on 33 patients (11 suggestive of malignant potential), Kang *et al*[36] failed to find features able to predict SPT malignant behavior in the clinical setting, although both patients with metastasis and recurrence belonged to a patient group with histopathology suggesting malignant potential. Thus, all patients require long-term follow-up.

In our patient, several different regimens were used. However, we do not know if the long term survival is due to the chemotherapy, or due to the natural course of a less aggressive tumor. The usefulness of chemotherapy for patients with SPT is substantially unknown, although some anecdotal studies have reported its benefit[11,31,40,41] or non benefit[10,17,36]. Two cases of resectable tumor underwent chemotherapy with cisplatin and 5-fluorouracil[40] or gemcitabine[41], but in the latter patient previous treatment with fluorouracil and 50.4 Gy failed to decrease the tumor size. Radiotherapy is seldom used for unresectable tumor[11] or as an adjuvant after tumor resection[8]. Two cases of radio sensitivity in unresectable tumors[9,26] are reported.

Transcatheter arterial embolization (TAE) or chemoembolization (TACE) are seldom used[10,30,36]. One patient was reported to have significant reduction metastases in the right lobe[10], one died after the procedure[30], one remained unchanged[36]. Radiofrequency is also seldom used[35,36].

In conclusion, malignant SPT is a low grade tumor with a good prognosis. Adequate surgical intervention is necessary for the long term survival of patients. Whenever possible, aggressive and reiterative surgery is justified for locally invasive or metastatic SPT. Regular follow-up is mandatory for all patients having undergone potentially curative resection. Particular attention should be paid to the patients with tumors larger than 5 cm in diameter and/or with histology suggesting a possible malignant behavior. The role of chemotherapy, radiotherapy, TAE, TACE, radiofrequency is still under scrutiny. Careful follow-up also for unresectable pancreatic cystic lesions may prevent tumor progression.

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CASE REPORT

Colonic duplication in an adult mimicking a tumor of pancreas

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Abstract

Duplications of the alimentary tract are uncommon congenital malformations that can present diagnostic difficulties. We report a rare case of a cystic colonic duplication in a female adult. Preoperative investigations were suggestive of pancreatic tumor. The diagnosis was established based on the histopathological examination of the resected specimen. We concluded that, though uncommon, intestinal duplication should be considered in differential diagnosis of abdominal mass.

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Key words: Intestinal duplication; Ectopic gastric mucosa; Pancreatic tumor; Congenital malformations; Abdominal mass

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INTRODUCTION

Duplications of the alimentary tract are uncommon congenital malformations that can occur anywhere in the tract, and are most commonly detected in childhood. Depending on the age of patient, localization, size and shape of the duplications, they may present a diagnostic challenge[1]. We report a rare case of a cystic colonic duplication in an adult mimicking a tumor of the pancreas.

CASE REPORT

A 43-year-old woman was admitted with a 2-mo history of abdominal pain and mass. Ultrasonography (US) and computerized tomography (CT) showed a cystic mass (Figure 1) in left upper abdomen. In laboratory studies, serum amylase level was elevated at 332 U/L (normal, 0-125 U/L). The preoperative diagnosis was pancreatic tumor and fine needle aspiration biopsy was not performed preoperatively. At laparotomy, a mass was found in the mesenteric border of the transverse colon attached to the colon and pancreas. A distal pancreatectomy was performed with resection of the cyst. On gross examination, the cyst was 6 cm in diameter and contained a serous fluid (Figure 2). Microscopically, the cyst was lined by ulcerated gastric mucosa, submucosa and muscular wall (Figure 3); pancreatic tissue was normal. The patient's pain improved following surgical resection of the mass.

DISCUSSION

Duplications of the alimentary tract are characterized by attachment to at least one part of the tract, a layer of smooth muscle and epithelial lining resembling some part of the tract[2]. These malformations are formed before differentiation of the lining epithelium, and therefore, are named depending on the associated part of the bowel and not according to the mucosal lining[3]. Gastric mucosa, for example, is frequently observed in the wall of duplications, irrespective of their site of origin in the alimentary tract[4]. The duplications are located on the mesenteric border of the associated native bowel, vary in size and shape (cystic or tubular) and communicate or not with the lumen of the adjacent bowel; the duplication of cystic type usually does not communicate with the lumen of the adjacent bowel and contains serous fluid[1]. In the present case, the duplication was cystic and there was no communication with lumen of the colon.

The diagnosis of intestinal duplication may be difficult and usually is not made preoperatively. The patient may remain asymptomatic or present with a variety of nonspecific signs and symptoms[5]. Similar to our case, these signs and symptoms usually include abdominal mass and pain, often confusing colonic duplication with

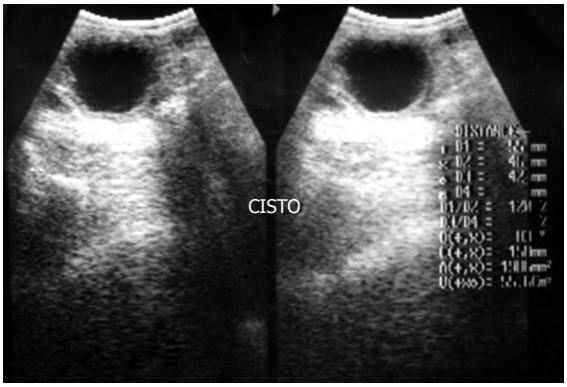


Figure 1 Ultrasonography demonstrating a cystic lesion.

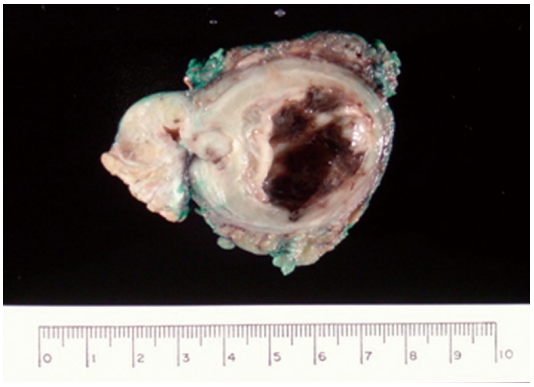


Figure 2 Surgical specimen including distal pancreas and the cyst.

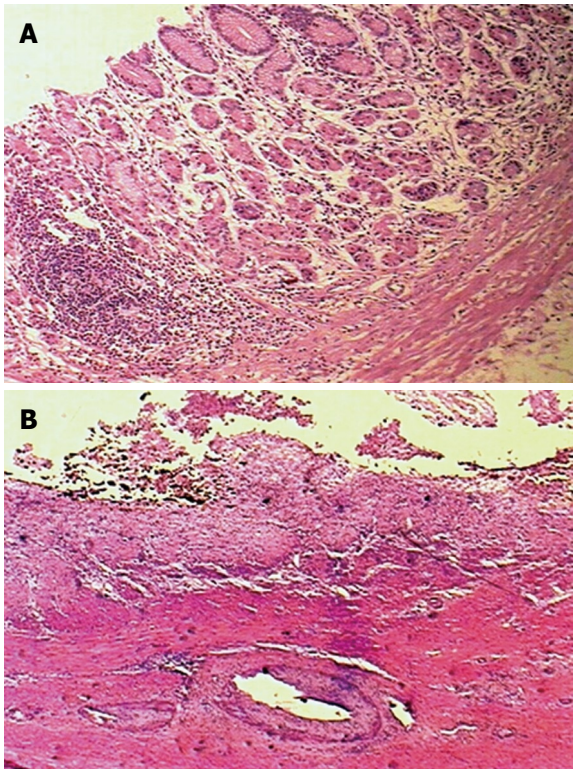


Figure 3 Histological section of the cyst wall. **A**: Ectopic gastric mucosa with gastritis (HE, x 100); **B**: Ectopic gastric mucosa with ulceration (HE, x 25).

other diagnoses. Intestinal obstruction[6], bleeding[7] and malignant transformation[8] are all recognized complications. Ulceration, perforation and peritonitis may also occur in cases with ectopic gastric mucosa[9], which is probably the cause of ulceration in our case. In addition, various urogenital and vertebral malformations have been reported but, in the present report, no associated congenital abnormality was observed[5].

In our case, elevated serum amylase, probably owing to compression of the pancreatic duct, associated with the findings of US and CT were suggestive of pancreatic cyst, but some image findings may be helpful in establishing preoperative diagnosis of intestinal duplication. On US, the duplication is characterized by "gut signature" which refers to the hiperechoic inner layer produced by the mucosa surrounded by a hypoechoic outer layer caused by smooth muscle[10]. Peristalsis of the cyst wall has also been noted on ultrasound scan[11]. Contrast barium enema may show communication between the duplication and the normal bowel, with opacification of the two colons[12]; the communication between the duplication cyst and main pancreatic duct may be seen by endoscopic retrograde cholangiopancreatography[13]. Besides this, technetium scan may demonstrate ectopic gastric mucosa in duplication[14].

Previous reports[15-17] have described gastrointestinal duplications mimicking pancreatic tumor. Among them, a case of transverse colon duplication in a child, like in our case, was not suspected before surgery[18]. The differential diagnosis includes in these cases, all neoplastic and non-neoplastic cystic lesions of the pancreas as intraductal papillary-mucinous neoplasm and pseudocyst[19].

The recommended treatment for duplication is complete resection. Sometimes resection of normal intestine must accompany removal of the lesion because of the intimate attachment of the common wall or because isolated resection would compromise blood flow to the adjacent intestine segment[20]. Generally, duplications of the cystic type are easily removed from their attachments to the surrounding tissues[21]. During surgery, diagnosis may be made based on the frozen sections examination of the wall of the duplication[22] but, in our case, it was not performed.

On histopathological examination, ectopic gastric mucosa showed chronic inflammation, lymphoid follicles and ulceration but no *H pylori* was identified in haematoxilin-eosin and Warthin-Starry silver stains. Previous reports about the role of *H pylori* in complications of the ectopic gastric mucosa in intestinal duplication were not found. But in most cases of ectopic gastric mucosa in Meckel's Diverticulum, *H pylori* also is not identified and seems that its presence is not essential to induce "heterotopic gastritis" and its complications[23-25]. In our case, the endoscopic examination was normal, and gastric biopsy was not performed.

In conclusion, we reported a case of cystic colonic duplication in an adult and illustrated the difficulties in making this rare diagnosis that should be considered in differential diagnosis of abdominal mass.

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CASE REPORT

Synchronous colorectal and lung cancer: Report of three cases

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Abstract

The incidence of synchronous colorectal and lung cancer is relatively rare. We report three cases of patients with tumors located in the rectum, ascending colon, the lower lobe of the left lung, and the upper lobe of the right lung. Synchronous curative resection of the two lesions was performed in two patients, whereas colectomy was performed in an elderly patient with a poor lung function. Pathological examination showed the colorectal cancer was a moderately differentiated adenocarcinoma and the lung cancer was a squamous cell carcinoma. Surgical treatment and postoperative adjuvant chemotherapy for the lung cancer were different from those for colorectal cancer with pulmonary metastasis. If possible, radical resection should be performed for each cancer when synchronicity is found.

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Key words: Multiple primary malignancies; Adenocarcinoma; Squamous cell carcinoma

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INTRODUCTION

Curative resection is regarded as a safe and effective treatment for colorectal cancer. The liver is the first and most common site of colorectal cancer metastases. Because of its abundant circulation, the lung is the second most common site of metastases[1]. Coya *et al*[2] reported that lung metastases develop in 10%-20% of patients with colorectal cancer. Using medical imaging methods (e.g. CT scan), pulmonary metastasis of colorectal cancer almost always shows multiple lesions in both lungs because of the distribution of primary tumor via the circulation. However, about 10% of pulmonary metastases consist of a single solitary pulmonary nodule[2]. When a patient with a current or previous colorectal cancer develops a solitary pulmonary nodule, surgeons always judge it to be a metastasis of colorectal cancer in the first instance. However, in some rare cases, patients can develop synchronous colorectal and lung cancer. In these patients, not only surgical resection but also chemotherapy regimens are quite different from those for colorectal cancer patients with pulmonary metastasis. Therefore, effectively distinguishing synchronous cancers from colorectal cancer with pulmonary metastasis has important implications in therapy for these patients. Although it is reported rarely, three patients with synchronous colorectal and lung cancer were admitted to our department between September 2004 and February 2006.

CASE REPORT

Case 1

A 63-year-old female patient presented with blood in stools for 4 mo. She had no prior family history of malignancy and no record of carcinogen exposure. A polypoid mass was found 4 cm from the anal verge at colonoscopy. Biopsy of the mass revealed a moderately differentiated adenocarcinoma. CT scan of the pelvic showed the thickening wall of rectum (Figure 1A). Serum carcinoembryonic antigen (CEA) was elevated to 7.9 ng/mL. No metastases were found in the abdomen or chest by CT scan. We performed low anterior resection, and microscopic examination of the surgical resection specimen showed a moderately differentiated adenocarcinoma (2 cm in diameter). The lesion infiltrated the proper muscular layer but not the lymph nodes or vessels. Immunostaining was positive for c-met, p53, p27, cd44, p170, ToPo-Ⅱ, and MMP-7. According to the National Comprehensive Cancer Network (NCCN) guidelines for rectal cancer, the tumor in this patient was classified as T2N0M0, and not treated with adjuvant chemotherapy after operation. Five months later during routine follow-up, serum CEA was elevated to 28.9 ng/mL. CT scan of the chest showed an irregularly shaped solid mass (3.5 cm in diameter) in the lower lobe of the left lung (Figure 1B), which was an adenosquamous cell carcinoma confirmed by biopsy. The mass in the lung was diagnosed as a metastasis of rectal cancer and a lower lobectomy of the left lung was performed. Microscopic

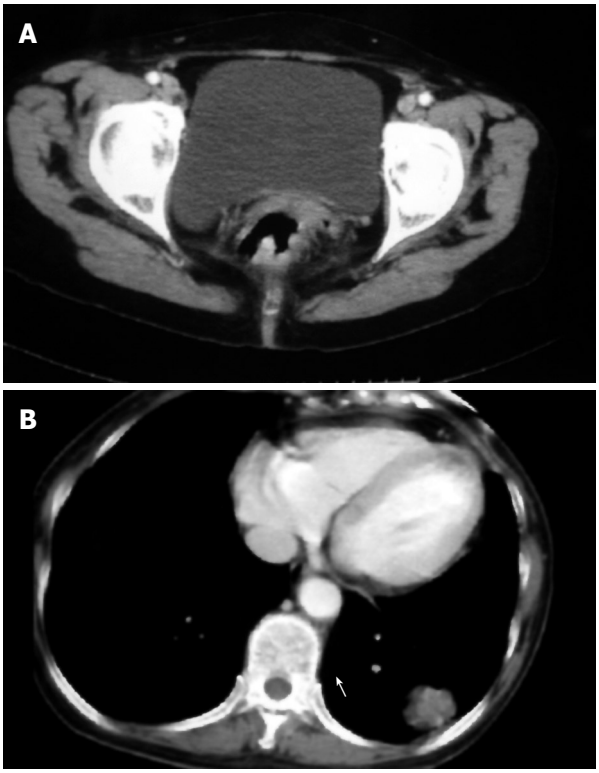


Figure 1 CT scan of the pelvic (**A**) and chest (**B**) showing the thickening wall of rectum and an irregularly shaped solid mass (3.5 cm in diameter) in the lower lobe of the left lung of case 1.

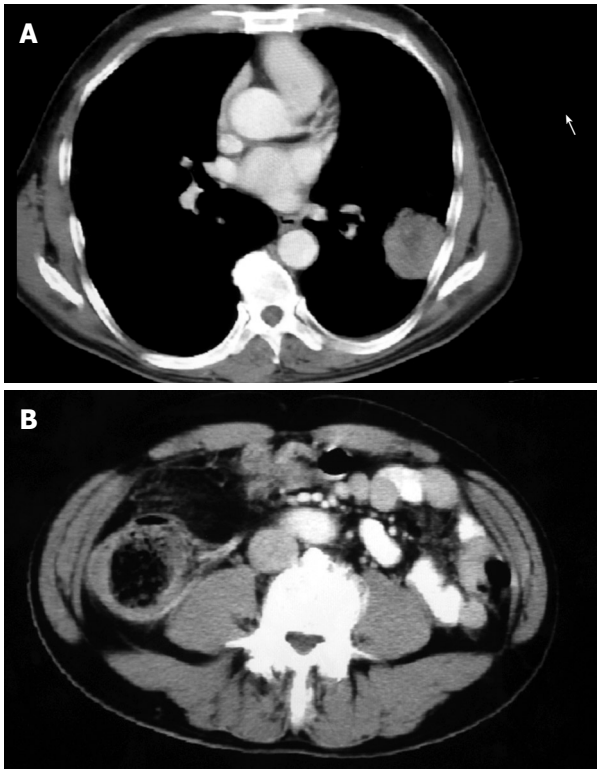


Figure 2 CT scan of the chest (**A**) and abdomen (**B**) showing an anomalous round solid mass in the lower lobe of the left lung and an intraluminal mass in the ascending colon with an irregular and stenotic lumen in case 2.

examination of the surgical resection specimen showed an adenocarcinoma (3 cm in diameter) with neuroendocrine differentiation. The lesion invaded the lymph nodes (7/13) and vessels. Immunostaining was positive for CK, EMA, and Syn, but negative for Vim. The final diagnosis was a primary lung cancer. Eighteen months after resection of the lung cancer, the patient died of multiple metastases of the lung cancer.

Case 2

A 60-year-old male patient was admitted for irritant cough in the previous three months. He had no family history of malignancy and alcohol or cigarette abuse, and his dietary habits were normal. CT scan of the chest showed an anomalous round solid mass in the lower lobe of the left lung (Figure 2A). The density of the mass was not uniform, and its margin was blurred. The mass was diagnosed as a primary lung cancer, and we decided to perform a radical lobectomy of the left lung. However, routine B-ultrasonography of the abdomen showed a mass in the right lower abdomen, colonoscopy revealed a polypoid mass in the ascending colon, obstructing the lumen, and a biopsy revealed a moderately differentiated adenocarcinoma. A CT scan of the abdomen showed an intraluminal mass in the ascending colon, the lumen was irregular and stenotic (Figure 2B). Serum CEA was elevated to 7.5 ng/mL. We diagnosed it as colon cancer with metastasis of the lung, and performed a right colectomy and lower lobectomy of the left lung on the same day (Figures 3A and 3B).

Pathological examination of the mass from the colon revealed a moderately differentiated adenocarcinoma, which

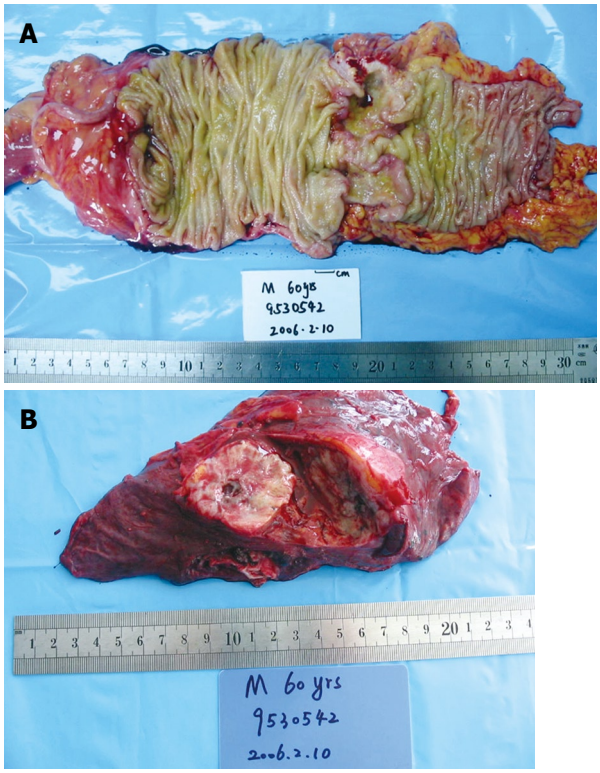


Figure 3 Surgical specimens of the colon cancer (**A**) and ascending cancer (**B**) from case three.

infiltrated the serosa but not the vessels or lymph nodes (0/12). Immunostaining was positive for CEA, CK20, p53,

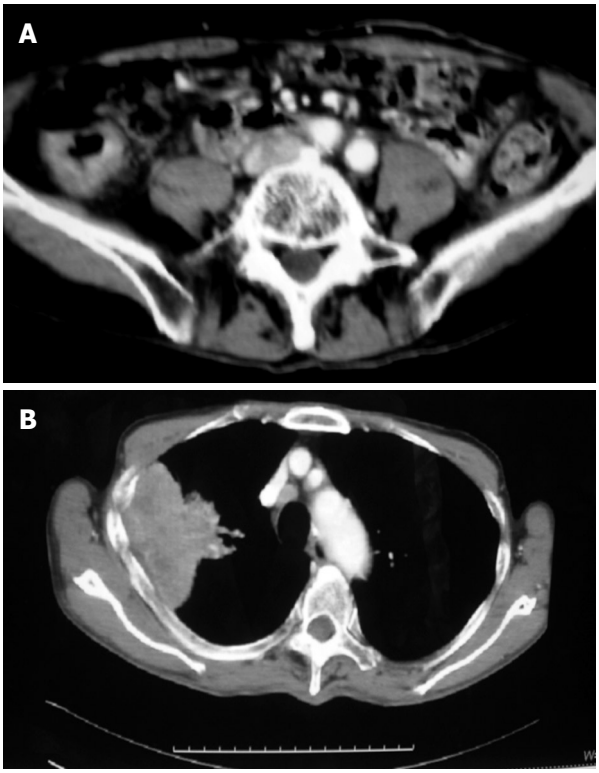


Figure 4 CT scan of the abdomen (**A**) and chest (**B**) showing a mass in the ascending colon and an irregularly shaped mass in the upper lobe of the right lung.

CDX-2, p27, p170, and Topo-Ⅱ, while negative for CK7, CD44, and TTF-1.

Pathological examination of the mass (3 cm in diameter) from the lung also revealed a moderately differentiated adenocarcinoma, invading the pleura and lymph nodes (1/11) but not the vessels. Immunostaining was positive for CEA, CK7, p53, CDX-2, and TTF-1, while negative for CK20. The final diagnosis was synchronous colon and lung cancer. After operation, the patient received chemotherapy (3 wk/cycle) with taxol (75 mg/m^2) and oxaliplatin (100 mg/m^2) on d 1, with a total of 6 cycles. The patient was still alive 11 mo after operation.

Case 3

An 81-year-old male patient had heme-positive stools for three months, and was difficult to pass stools for one week. He had no family history of malignancy and alcohol or cigarette abuse. Physical examination was normal, colonoscopy showed an ulcerous mass in the ascending colon, involving the hemi-cycle of the lumen, a biopsy revealed a moderately differentiated adenocarcinoma. B-ultrasonography of the abdomen showed multiple lesions in the liver, and a diagnosis of colon cancer with metastasis of the liver was made. CT scan of the abdomen showed a mass in the ascending colon (Figure 4A). A chest X-ray before operation demonstrated a mass in the upper lobe of the right lung. CT scan of the chest revealed an irregularly shaped mass (5 cm in diameter) in the upper lobe of the right lung, invading the pleura, enlarged mediastinal and hilar lymph nodes (Figure 4B). A CT-guided fine needle biopsy of the mass revealed a poorly differentiated squamous cell carcinoma. Serum CEA was 1.53 ng/mL, CYFRA21-1 and NSE were elevated to 22.90 ng/mL and 14.32 ng/mL. A pulmonary function test showed significant loss of ventilatory function. Since the patient could not tolerate a lobectomy of the lung and had incomplete obstruction of the colon, right hemi-colectomy and biopsy of the lesion on the liver were performed (Figure 5). Pathological examination of the colonic mass (4 cm in diameter) indicated a moderately differentiated adenocarcinoma, invading the serosa but not vessel or lymph nodes (0/5). The liver biopsy revealed a metastatic adenocarcinoma. Immunostaining was positive for c-met, p53, p27, p170, and Topo-Ⅱ, while negative for CD44. The final diagnosis was colon cancer with multiple metastases in the liver and synchronous primary squamous cell lung cancer. After operation, the patient received chemotherapy (3 wk/cycle) with taxol (175 mg/m^2) on d 1, 5-fluorouracil (5-FU) (500 mg/m^2) and CDDP (20 mg/m^2) on d 1-5, for 3 cycles. The primary lung cancer and liver metastases of the colon cancer were controlled well. The patient was still alive 8 mo after the operation.



Figure 5 Surgical resection specimens of the ascending colon.

DISCUSSION

Colorectal cancer is the most common cancer, and also most prone to blood-borne metastasis. The liver and lung are the most common sites of metastasis. About 50% of patients develop liver metastases and about 20% develop lung metastases. Therefore, when multiple pulmonary lesions are found in patients with a history of colorectal cancer, most physicians consider them as lung metastases. However, sometimes pulmonary metastases show up as a single solitary lesion. There are a few reports on colorectal cancer with primary lung cancer as a type of multiple primary malignant neoplasm (MPMN) the references is just the Evans *et al*[3]. Evans *et al*[3] investigated the incidence of primary lung cancer in 127 281 patients with colorectal cancer and found that the incidence of primary lung cancer is 0.6% (801 cases), while the incidence of synchronous colorectal and lung cancer is much lower. The pathogenesis and biological behavior of synchronous colorectal and lung cancer *vs* colorectal cancer with pulmonary metastasis are quite different, and so is their treatment. Therefore, when a single solitary pulmonary lesion is found in a colorectal cancer patient, it is important to distinguish between these two possibilities

for their appropriate surgical treatment and postoperative adjuvant chemotherapy.

Because of the rare incidence, physicians lack the knowledge of and vigilance towards synchronous colorectal and lung cancer. This situation may lead to misdiagnosis and delay in treatment. When synchronous colorectal and lung cancer are misdiagnosed as colorectal cancer with pulmonary metastasis, adjuvant chemotherapy for colorectal cancer is very unfavorable for the patient.

In our group, two male patients admitted to our department were initially suspected of having a tumor in a single organ. The 60-year-old male patient complained of irritating cough. CT scan revealed a mass in the lower lobe of the lung. Pre-operative abdominal ultrasound examination accidentally found the mass in the right lower abdomen because there were no clear abdominal symptoms, colonoscopy revealed a mass in the ascending colon and adenocarcinoma was confirmed by biopsy. The 81-year-old male patient was admitted to hospital because he felt weak and emaciated with a palpable mass in the right lower abdomen for one week. Colonoscopy found a mass in the ascending colon, which was confirmed as an adenocarcinoma by biopsy. However, conventional preoperative chest radiography showed a suspicious mass in the left upper lobe of the lung, which was confirmed as a squamous cell carcinoma by CT guided fine-needle aspiration. In the female patient with rectal cancer who received the radical LAR procedure, a moderately differentiated adenocarcinoma was confirmed by postoperative pathology. Five months after the operation, conventional chest radiography revealed a nodule in the left lung, which was considered a lung metastasis of rectal cancer and a lower lobectomy of the left lung was performed. The pulmonary nodule was a differentiated adenocarcinoma with neuroendocrine differentiation confirmed by pathology. In the three patients, the symptoms of secondary primary cancer found by conventional preoperative examination or postoperative review were relatively unobvious, suggesting that although the incidence of MPMN is relatively low, when the first primary cancer is found, the surgeon cannot simply be satisfied with a single diagnosis and meticulous systemic examinations of the patient are necessary to avoid misdiagnosis. Further examination of the suspicious lesions should be conducted. If necessary, biopsy can confirm the diagnosis.

When colorectal and pulmonary lesions are found synchronously in a patient, how to distinguish between the pulmonary lesion and primary cancer or the metastasis of colorectal cancer warrants attention.

First, a medical history is helpful for judging the characteristics of pulmonary lesions. Quint *et al*[4] evaluated 149 patients with extrapulmonary malignant neoplasm and solitary pulmonary nodule and found that a single solitary pulmonary nodule is more likely diagnosed as primary lung cancer than metastasis of other cancers in elderly patients or patients with a history of smoking. They also evaluated the characteristics of solitary pulmonary nodule in patients with a history of colorectal cancer and found the proportion of primary lung cancer and metastases of other cancers is statistically identical. Furthermore, they found that the longer the interval between the detection of primary colorectal cancer and solitary pulmonary nodule is, the more likely the diagnosis of primary lung cancer is. However, in our study, the three patients were non-smoker, and the interval between the detection of colorectal and lung cancer was less than six months.

Second, Quint *et al*[4] reported that analysis of nodule morphology may provide important information on the lesion etiology and a mass with a smooth margin can be an indication of a benign lesion or a metastasis, whereas a mass with an irregular or blurry margin may be theoretically suggestive of a new lung cancer. Except for patients with melanoma, sarcoma, and testicular carcinoma, the presence of mediastinal lymph node enlargement is strongly suggestive of a primary lung cancer because lymph node enlargement results from metastasis of a primary lung cancer. In our group, the margins of solitary pulmonary nodules in the three patients were all blurry and irregular. On this point, our results are in agreement with the literature reports. However, we found that only one patient had enlarged mediastinal lymph nodes, the other two did not.

Third, sometimes, the characteristics of a pulmonary nodule in a patient with colorectal cancer cannot be determined before operation. In this case, pathological examination of the surgical resection specimen becomes crucial. For example, tumors of different histological findings (e.g. carcinoma and sarcoma) may develop in the same patient. The diagnosis of synchronous MPMN is then without argument. In the 81-year-old male patient, pathology showed the rectal lesion was an adenocarcinoma, whereas the pulmonary lesion was a squamous cell carcinoma. In the female patient, pathology revealed that the rectal lesion was a moderately differentiated adenocarcinoma, whereas the pulmonary lesion was an adenocarcinoma with neuroendocrine differentiation invading the vessels and lymph nodes. Thus, a diagnosis of MPMN was conclusive for these two patients.

However, when similar histological results (e.g. adenocarcinoma) are found in colorectal and pulmonary lesions, the question is whether both lesions are primary cancer or the pulmonary lesion is a metastasis of the colorectal cancer. Microscopy of a conventional pathological section is not enough. Kummer *et al*[5] used immunohistochemical staining as an effective differential diagnosis method because primary lung cancer shows a CK7(+)/CK20(-) cytokeratin pattern while a metastatic adenocarcinoma shows a CK7(-)/CK20(+) pattern, and found that in their 102 tumor samples, the CK7/CK20 pattern was able to identify lung and colorectal adenocarcinoma in 95% of cases, suggesting that cytokeratin staining is a cost-effective screening tool. Others have reported the use of thyroid transcription factor-1 (TTF-1) for distinguishing between primary and metastatic lung adenocarcinomas[6]. TTF-1 is a common thyroid and peripheral lung antigen, positively stained in primary lung and thyroid adenocarcinoma, whereas metastatic adenocarcinomas from other sites do not express TTF-1. It was reported that over 75% of primary pulmonary adenocarcinomas show strong staining for TTF-1 and a further 11% show weak staining. In their series, apart from a new neuroendocrine tumor, none of the 106 non-

pulmonary adenocarcinomas showed staining for TTF-1, indicating that TTF-1 staining is useful in the differential diagnosis of pulmonary adenocarcinoma. The pathology of our 60-year-old male patient showed that colon and pulmonary lesion were a moderately differentiated adenocarcinoma. Therefore, immunohistochemical staining was performed for CK7, CK20 and TTF-1, to distinguish between primary and metastatic lung adenocarcinoma. We found that adenocarcinoma of the colon showed a CK7(-), CK20(+), TTF-1(-) pattern, whereas adenocarcinoma of the lung showed a CK7(+), CK20(-), TTF-1(+) pattern, indicating that it is a primary tumor. Recently, Takashi *et al*[7] reported that protein TA02 (napsin A), detected by two-dimensional polyacrylamide gel electrophoresis (2-DE), is a valuable marker for discriminating between primary and metastatic lung adenocarcinomas. Using monoclonal antibody to TA02, immunohistochemical staining for TA02 has greater specificity and sensitivity in distinguishing a primary from a metastatic lung adenocarcinoma than TTF-1.

Surgical treatment for patients with synchronous colorectal and lung cancer is quite different from that of colorectal cancer patients with pulmonary metastasis. Pulmonary metastases of colorectal cancer are always multiple lesions in both lungs which are unresectable. Chemotherapy or other palliative therapy is the first choice of treatment for such patients. Curative resection is performed only for patients with single or a few pulmonary metastatic lesions. For patients with synchronous colorectal and lung cancer, if the condition of the patient permits, synchronous curative resection of the two lesions is the first choice. Patient with a poor general state cannot tolerate synchronous curative resection, and the surgeon should first deal with the tumor with a poor prognosis that directly endangers the patient life.

According to the NCCN guidelines, 5-FU and leucovorin combined with oxaliplatin (FOLFOX) or CPT-11 (FOLFIRI) are the first-line chemotherapy regimens for colorectal cancer[8], and cisplatin or carboplatin combined with taxol or docetaxel (TP) or gemcitabine (GP) is the first-line chemotherapy regimen for non-small cell lung cancer[9]. Because few cases of synchronous colon and lung cancer have been reported, a postoperative chemotherapy regimen has not been explicitly specified.

After surgical resection of the synchronous colon and lung cancers in our 60-year-old male patient, we discussed the chemotherapy regimen with the pathologist and oncologist. According to the type of the two primary tumors and stages of pathology, with reference to the NCCN treatment guidelines, a combination of both chemotherapy regimens for primary tumor, a 3-wk/cycle regimen with taxol (175 mg/m^2) and oxaliplatin (100 mg/m^2) on d 1 was used. After six cycles, no recurrence was found in this patient.

Literature reports indicate that patients with MPMN have a better prognosis than those with a metastasis or recurrent lesion. Tichunsky *et al*[10] reported that when surgical resection combined with postoperative chemotherapy is performed for colorectal cancer patients with a secondary malignant tumor in another organ, the five-year survival rate is 49%.

Since our 83-year-old male patient had a poor lung function and was unable to tolerate lobectomy of the lung, only colectomy was performed. The other two patients underwent radical resection of both primary tumors. The female patient died of multiple metastases 18 mo after operation, while the 83-year-old male patient with a lung tumor survived for 12 mo and the 60-year-old male patient survived and was disease-free for 13 mo after operation.

Therefore, careful examination, early detection of multiple primary cancers, and active surgical approach can improve the therapeutic effects in MPMN patients.

In conclusion, the incidence of synchronous colorectal and lung cancer is relatively low. When the first primary cancer is found, a meticulous examination of every organ should be conducted until no other malignant neoplasm is found. When suspicious lesions are found, further examination should be performed immediately. If necessary, biopsy can reduce the probability of misdiagnosis and rule out metastatic lesions.

Synchronous radical resection should be performed when the condition of the patient permits. After radical resection, according to the pathological type and stage of the tumors, appropriate adjuvant chemotherapy should be given. The prognosis of synchronous colon and lung cancer is relatively good after careful treatment.

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CASE REPORT

Graft-verse-host disease after liver transplantation: A report of two cases and review of literature

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Abstract

Graft-verse-host disease (GVHD) is an uncommon fatal complication following liver transplantation (LTx). In mainland China, only six cases have been reported with a morbidity rate up to 1%-2%. Definitive diagnosis was achieved by molecular techniques (HLA typing or PCR-STR) in only two cases and the remaining cases were diagnosed based on typical clinical features with exclusion of other possible causes. All patients died of septic shock or multiple organ failure even after administration of increased corticosteroids and supportive therapy, and reduced immunosuppressive agents. In our center, two cases of GVHD were found among 128 (1.56%) patients. One case was diagnosed by detecting lymphocyte macrochimerism through DNA-STR. Both of them died even after aggressive treatment. In China, the incidence of GVHD is similar to that reported by foreign centers except for an extremely bad prognosis. Rapid diagnosis is crucial for a better prognosis. In China, only 37.5% of cases are diagnosed by molecular methods. We recommend detecting lymphocyte macrochimerism through DNA-STR to get a rapid diagnosis, and interleukin 2-receptor antibody (basiliximab or daclizumab) therapy seems to be a good choice for the disease.

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Key words: Graft-versus-host disease; Liver transplantation; Prognosis; Diagnosis; Treatment

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INTRODUCTION

The rapid increase of liver transplantation (LTx) and the development of immunosuppressive agents are followed by an increased incidence of graft-verse-host disease (GVHD) after liver transplantation. GVHD is a rare but extremely severe complication from LTx. The incidence is about 1%-2% and the mortality rate over 75%[1,2]. Although the total number of LTx performed in 2005 reached 3500 in mainland China, less than 10 cases of GVHD after LTx have been reported and many cases were under-diagnosed because of poor clinical awareness and no qualified diagnostic methods. We review in this article the literature on the disease in China, including the incidence, clinical features, diagnostic methods, treatment and prognosis and report about our own experience on GVHD after LTx. This study was approved by the ethical committee of our hospital and conforms to the ethical guidelines of Helsinki Declaration.

CASE REPORT

We reviewed the records of 124 adults and 4 pediatric LTx in our organ transplantation center from December 2005 to December 2006, and found two patients with OLT associated GVHD (an incidence rate of 1.56%).

Case 1

Case one is a 50-year-old man admitted for liver cirrhosis secondary to hepatitis B and hepatocellular carcinoma (HCC). Before admission, the patient received regional therapy for HCC, including ethanol injection and radio-frequency ablation. And he also received chemotherapy (arsenious acid injection 10mg QD for 4 d) just before the operation because his waiting time was longer than expected. He received a modified Piggy-back procedure and two doses of daclizumab on post-transplantation d 1 and d 4 for induction. Donor's blood type was identical

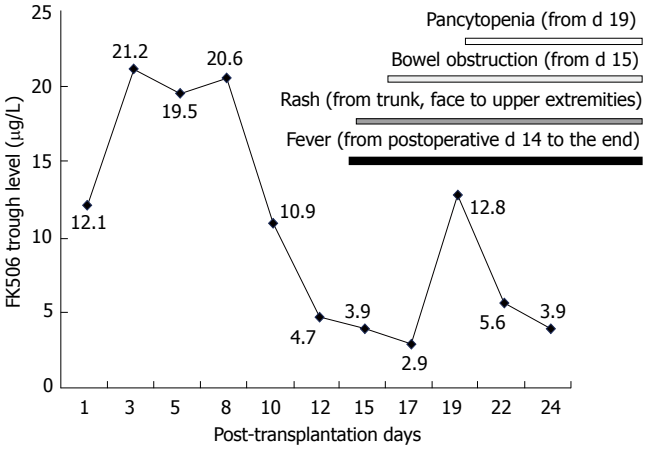


Figure 1 The relationship of FK506 trough level and the symptoms. Before the onset of disease, FK506 trough level was higher than the target level in case 1.

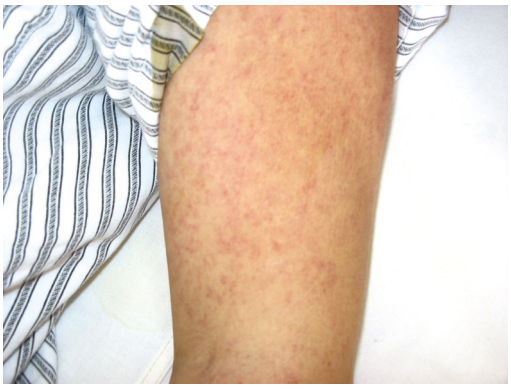


Figure 2 Rash on the right arm.



Figure 3 Pneumatosis intestinalis.

Table 1 STR system alleles of peripheral blood and normal skin after transplantation

STR alleles	Skin alleles	Peripheral blood alleles
Amelogenin	X, Y	X, Y
D5S818	10, 11	10, 11, 12
FGA	24, 24	23, 24, 25
D3S1358	15, 15	15, 15
TH01	9, 9	9, 9
D13S317	11, 14	8, 11,12, 14
D16S539	9, 11	9, 11, 14
D2S1338	24, 25	19, 24, 25, 26
D8S1179	10, 14	10, 13, 14
D21S11	30, 30	29, 30, 31
D7S820	10, 11	10, 11, 12
CSF1PO	10, 12	10, 12
D19S433	14.2, 15.2	14, 14.2, 15.2
vWA	18, 19	17, 18, 19
TPOX	8, 9	9, 11, 12
D18S51	20, 21	12, 13, 20, 21

STR: Short tandem repeat.

to the recipient's. Postoperative immunosuppressant regimen consisted of tacrolimus and methylprednisolone. FK506 trough level ranged from 2.9 μg/L to 21.2 μg/L (mean value 11.7 μg/L) (Figure 1). The patient had an uneventful early recovery and could take care of himself. Liver function tests (LFTs), electrolytes, blood film and ultrasonography were all normal. Discharge would be made if he had not complained of fever and headache. Remittent fever persisted until the patient died. Rash appeared one day after fever, so did the constipation. Eruption was maculopapular, accentuated the abdomen at the very beginning and then developed into the back and neck but not palms and toes (Figure 2), subsequently oral ulcer and sore throat occurred one week later. Considering the possibility of drug rash, we discontinued all suspected drugs and gave an injection of dexamethasone. A biopsy of skin showed no classical pathological change of GVHD. The patient also presented with nausea and vomiting without gas and stool passing. The abdominal film revealed lower incomplete intestinal obstruction (Figure 3). Nasogastric suction was used without improvement. On post-transplantation d 40, upper gastrointestinal bleeding developed with severe nausea and vomiting. Pancytopenia appeared suddenly with white blood cell count dropping from 4500/μL to 330/μL within one day, platelet 170 000/μL and hemoglobin 77 g/L. Bone marrow cellular study showed aplastic anemia. We suspected GVHD and a blood sample was sent to laboratory for DNA PCR-short tandem repeat (PCR-STR) which confirmed the diagnosis (Table 1 and Figure 4).We initiated G-CSF injection (Filgrastim 300 μg BID) and increased steroids dosage (methylprednisolone 100 mg QD for 6 d, then tapered gradually). Broad-spectrum antibiotics, anti-fungals and IVIG were also used to prevent infection. Unfortunately, the patient died of sepsis and multiple organ failure 19 d after the first onset of disease. Figure 5 shows the post-transplantation clinical course.

Case 2

Case 2 is a 56-year-old man with end-stage liver disease secondary to HBV infection. He received chemotherapy preoperatively for tuberculosis infection of chest wall for 6 mo. Modified Piggy-back liver transplantation was performed and he was treated with the immunosuppressive agents tacrolimus plus corticosteroids. The patient had an initial uneventful recovery in post-transplantation first week with LFTs dropping to the normal range. From postoperative d 8, the patient suffered from acute rejection which rapidly recovered in 10 d after MMF and corticosteroid impulse therapy was added. The patient experienced unidentified remittent fever without chill,

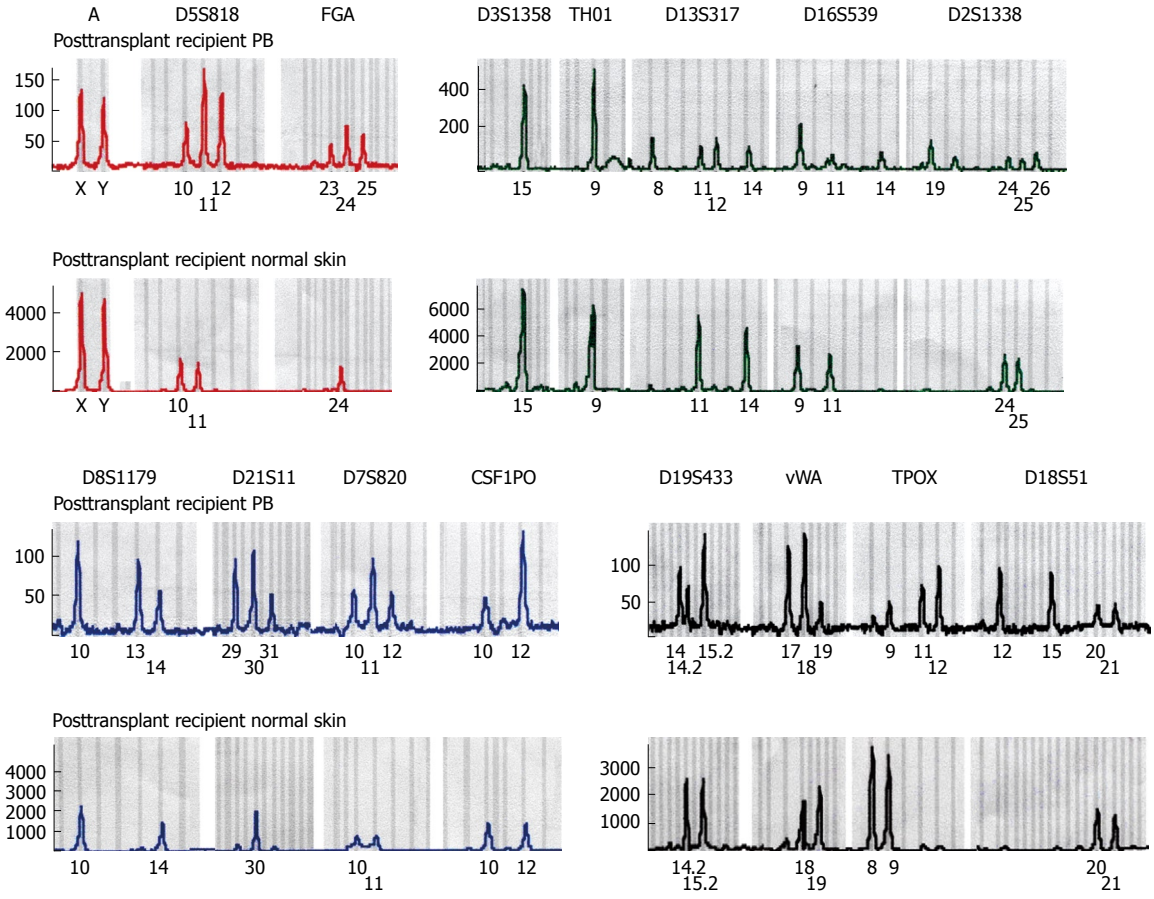


Figure 4 DNA-STR results of peripheral blood and normal skin in the transplanted recipients. The X axis corresponds to the size of amplified products as noted in base pairs. The Y axis illustrates the amount of fluorescent PCR products. Donor alleles are visible on makers D5S818, FGA D13S317, D16S539, D2S1338, D8S1179, D21S11, D7S820, D19S433, vWA, TPOX, D18S51.

accompanied by severe diarrhea, and maculopapular rash in face and neck. Since the patient had a history of tuberculosis infection, disseminated tuberculosis was suspected and he was started on chemotherapy. Multiple TB culture of urine and sputum (over 10 times respectively) and repeated bacterial culture and fungal culture of urine and peripheral blood showed no pathogen. Both CMV-Ag and EB-IgA were negative. And pancytopenia developed one week later. Then drug reaction and GVHD were suspected. The disease progressed even after withdrawing all suspected drug, corticosteroids therapy and supportive therapy were added, including broad-spectrum antibiotics, anti-fungals, G-CSF (Filgrastim 300 μg bid) and IVIG. Gastrointestinal bleeding occurred on post-transplantation d 59 and the patient died of pneumonia and multiple organ failure 5 d later. No definitive diagnosis was made at the end, however, GVHD was highly suspected because: (1) the clinical features were characteristic; (2) All symptoms started from post-transplantation wk 3, and no pathogen was found in the clinical course; (3) the disease progressed after withdrawing all suspected drugs; and (4) LFTs were within normal range when the disease developed and deteriorated at the end of clinical course.

DISCUSSION

GVHD is an increasing common fatal complication after LTx. The reported incidence of this complication varied from 0.1% to 2% with a high mortality rate of over 75%[1,2]. Its mechanism is still unclear although there are three known essential requirements[3]: (1) the graft must contain immunocompetent cells, (2) the recipient must be recognized as foreigner by the graft, and (3) the recipient must be unable to reject the graft before it mounts an effective immune response. A three-phase model is useful as a framework for understanding the pathogenesis of GVHD following LTx[4]. Phase 1 comprises the preparative regimen (chemotherapy and/or radiotherapy); phase 2 is the activation and proliferation of alloreactive donor T cells; and phase 3 is the effector phase. To date more and more research focuses on the cytokines network in the disease course. The clinical features consist of fever, rash, intestinal symptoms and neutropenia or pancytopenia which is undistinguished from drug reaction and viral infections. The diagnosis is based on the characteristic clinical presentations, histological proof of target tissue damage and molecular manifestation of invasion of recipient target tissue by donor T cells. The latter is most important because it is specific for GVHD and the remaining two may also be found in other diseases. The treatment regimen is generally disappointing, including corticosteroid therapy, reduced or increased immunosuppressant, prevention of infection and supportive treatment, although interleukin-2 receptor antibodies (basiliximab and daclizumab) are recommended

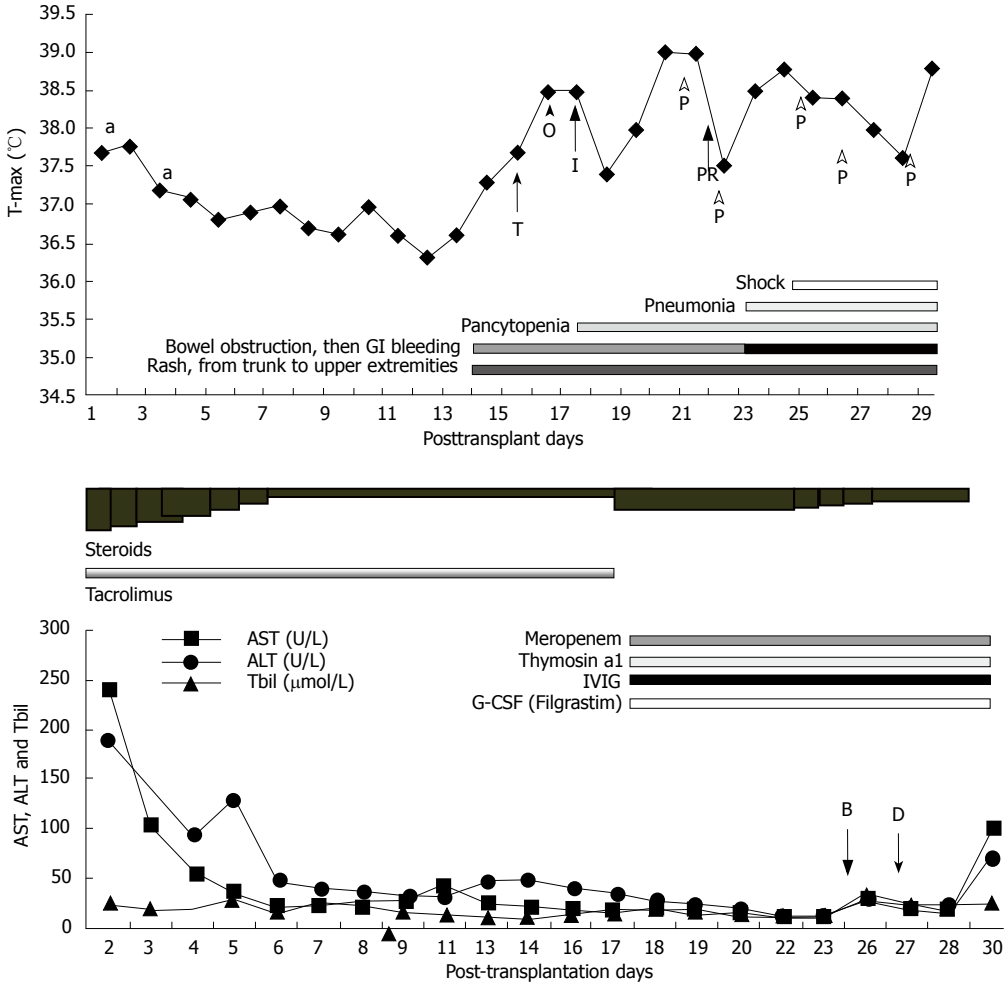


Figure 5 The posttransplant clinical course of case 1. a: Daclizumab, T: Tazime; O: Ornidazole; I: Isepamicin; P: Platelet; PR: Platelet and packed red blood cell; B: Biopsy of skin and bone marrow tapping; D: DNA-STR test of peripheral blood.

as an important treatment choice[5-7].

In a thorough search of Chinese language medical literatures and English language literatures by Chinese organ transplant institutions, we could find only two case reports by two different organ transplantation centers in mainland China (up to January 2007)[8,9]. Six cases with GVHD after LTx are summarized in Tables 2 and 3. Four cases were diagnosed by characteristic clinical presentation, the remaining two by PCR-SSP for detecting the peripheral lymphocyte macrochimerism. None of the six cases survived after empirical treatment.

The incidence and clinical presentations resemble that reported by others. We must take GVHD into consideration when a recipient presents with fever, rash, diarrhea and neutropenia or pancytopenia without known causes since the incidence of this disease is not so low as expected and the outcome is terribly bad. The diagnosis is difficult in the beginning of the clinical course, which poses a great challenge in treatment. In China, only 3/8 cases are diagnosed with specific molecular techniques which detect the lymphocyte macrochimerism or skin invasion by donor T cells, and 5/8 cases are diagnosed based on the classical clinical features with or without histology of rash skin biopsy and other causes. Without rapid molecular methods to confirm the diagnosis, early initiation of specific treatment is impossible and the poor outcome is unavoidable. No percentage of donor lymphocyte has been reported by other Chinese authors except our article, the so-called confirmed diagnosis may not be accurate because microchimerism is a common phenomenon within 3-4 wk after LTx[10]. The diagnosis needs to be supported by lymphocyte macrochimerism usually detected by HLA typing through PCR-SSP or by DNA PCR-STR with or without flow cytometric tests[11,12]. Fluorescent *in situ* hybridization (FISH) can also be used to confirm the diagnosis with limited function because unmatched gender is needed for this test. R.Domiati-Saad *et al*[13] compared different molecular techniques used for confirming the lymphocyte macrochimerism. They recommended: (1) Repeat HLA class I typing by serologic methods using purified T cells and/or class Ⅱ typing by PCR-SSP; (2) since PCR-SSP is too sensitive to normal levels, a level above 10% should be detected by serologic method, while PCR-SSP may detect levels around 1%; (3) STR method can be used to quantify the contribution of donor T cells when donor HLA antigens are detected, CD3 donor fraction above 20% is highly suggestive of GVHD; and 4) STR testing of CD3 or CD8 populations should be performed twice a week to monitor the patients' response to treatment. In our case 1, DNA-STR was used to detect lymphocyte macrochimerism. Lymphocytes of donor origin occupied over 60% of lymphocyte population and the diagnosis was confirmed although the patient did not have diarrhea and the histology of skin biopsy was not so clear. In our study, no patient survived after aggressive treatment. The extremely poor prognosis was attributed

Table 2 General data of the patients, onset of GVHD and diagnostic methods

Case	Gender	Age (yr)	Indication for LTx	Onset of GVHD	Diagnostic method
1	M	47	HCC	PD28 (rash)	Clinical diagnosis
2	M	58	HCC	PD27 (fever)	Clinical diagnosis
3	F	58	LC	PD25 (fever)	HLA typing by PCR-SSP
4	M	48	LC	PD21 (fever)	Clinical diagnosis
5	M	69	LC	PD21 (fever)	HLA typing by PCR-SSP
6	M	43	LC and HCC	PD27 (fever)	Clinical diagnosis

HCC: Hepatocellular carcinoma; LC: Lliver cirrhosis; HLA: Human leukocyte antigens; PCR-SSP: PCR-sequence specific primer; PD: Post-transplantation day.

Table 3 Treatment, time point of treatment and patient outcome

Case	Treatment	Time point of treatment after onset (d)	Outcome
1	Decreased immunosuppressant; G-CSF; IVIG	16	Death, infection
2	Decreased immunosuppressant; Methylprednisolone 80 mg × 10 d, then taper rapidly; G-CSF; IVIG	6	Death, gastrointestinal bleeding, infection, MOF
3	Decreased immunosuppressant; Methylprednisolone 80 mg × 10 d, then taper rapidly; G-CSF; ATG; IVIG	16	Death, gastrointestinal bleeding, infection, ARDS
4	Decreased immunosuppressant; Methylpredprednisolone impulse treatment, then taper; G-CSF	5	Death, gastrointestinal bleeding, infection, MOF
5	Discontinued immunosuppressant; Methylprednisolone impulse 480 mg/d, then taper; G-CSF; IVIG	6	Death, infection, gastrointestinal bleeding, respiratory failure
6	Non-specific treatment	?	Death, infection

G-CSF: Granulocyte colony-stimulating factor; IVIG: Intravenous immunoglobin therapy; MOF: Multiple organ failure; ARDS: Acute respiratory distress syndrome.

mostly to the failure in confirming early diagnosis and lack of specific treatment. The common treatment includes reducing or even withdrawing immunosuppressant, increasing corticosteroids and supportive therapy (broad-spectrum antibiotics, anti-fungals, antivirus, IVIG and G-CSF if neutropenia or pancytopenia develops). The dosage and duration of corticosteroids varied among different centers. No interleukin-2 receptor antibody (basiliximab or daclizumab) has been used as a treatment choice.

In summary, GVHD following OLT is under-diagnosed in China with a 100% mortality rate due to the poor awareness of the disease and lack of specific molecular techniques for diagnosis, and this is why we could only select the cases from 2005 to 2006. It is reported that HLA and age may increase the chance of GVHD development after OLT[1]. We believe that immunocompromise status of the recipients is important in the development of this disease because both of our patients received chemotherapy preoperatively. For the recipients suspected of having GVHD, we would suggest that: (1) when a recipient is presented with unidentified fever, rash, gastrointestinal symptoms and bone marrow failure, GVHD must be taken into consideration as the failed early diagnosis would endanger the patient with the edge of death; (2) biopsy of rash skin or damaged gut should be done as soon as possible, which can support the diagnosis of GVHD even though it is not specific for this disease. And HLA typing of both recipients and donors should be performed preoperatively so that lymphocyte macrochimerism can be detected by HLA typing through PCR-SSP or serologic methods. Quantified the contribution of donor T cells is crucial for establishing the diagnosis since lymphocyte microchimerism is a common phenomenon in the early stage after OLT. STR is a valid and rapid technique for this purpose; (3) when GVHD is highly suspected or confirmed and infection is ruled out, specific treatment should be initiated as soon as possible which includes corticosteroids, reduced immunosuppressive agents, prevention of infection and supportive therapy. Interleukin-2 receptor antibody (basiliximab or daclizumab) should also be tried since it has been reported to be successful in several cases.

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[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

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EDITORIAL

Neuro-regulation of lower esophageal sphincter function as treatment for gastroesophageal reflux disease

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Abstract

The junction between the esophagus and the stomach is a specialized region, composed of lower esophageal sphincter (LES) and its adjacent anatomical structures, the gastric sling and crural diaphragm. Together these structures work in a coordinated manner to allow ingested food into the stomach while preventing reflux of gastric contents across the esophago-gastric junction (EGJ) into the esophagus. The same zone also permits retrograde passage of air and gastric contents into esophagus during belching and vomiting. The precise coordination required to execute such a complicated task is achieved by a finely-regulated high-pressure zone. This zone keeps the junction between esophagus and stomach continuously closed, but is still able to relax briefly *via* input from inhibitory neurons that are responsible for its innervation. Alterations of the structure and function of the EGJ and the LES may predispose to gastroesophageal reflux disease (GERD).

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Key words: Gastroesophageal reflux disease; Lower esophageal sphincter; Esophago-gastric junction

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INTRODUCTION

The junction between the esophagus and the stomach is a specialized region, composed of the lower esophageal sphincter (LES) and its adjacent anatomical structures, the gastric sling and the crural diaphragm. Together these structures work in a coordinated manner to allow ingested food into the stomach while preventing reflux of gastric contents across the esophago-gastric junction (EGJ) into the esophagus. The same zone also permits retrograde passage of air and gastric contents into the esophagus during belching and vomiting. The precise coordination required to execute such complicated tasks is achieved by a finely-regulated high-pressure zone. This zone keeps the junction between esophagus and stomach continuously closed, but is still able to relax briefly via input from inhibitory neurons[1] that are responsible for its innervation. Alterations of the structure and function of the EGJ and the LES may predispose to gastroesophageal reflux disease (GERD) (Table 1).

ANATOMY, INNERVATION AND NEURAL PATHWAYS

At the EGJ, the LES and the crural diaphragm constitute the intrinsic and extrinsic sphincters, respectively. These two sphincters are anatomically superimposed and are anchored to each other by the phreno-esophageal ligament[2]. The LES is a specialized thickened region of the circular muscle layer of the distal esophagus, extending in humans over an axial distance of 2-5 cm. Sensory information from LES to the brain is conveyed by both spinal and vagal sensory afferents[3,4]. The spinal afferents have their cell bodies in the dorsal root ganglia at T1 to L3, and are believed to detect mainly nociceptive stimuli[5]. The vagal afferents, with their cell bodies in nodose ganglia, transmit non-painful sensory information to the brain stem where the central terminations synapse in the nucleus tractus solitarius[1]. The neurons of this nucleus are closely connected to dorsal motor nucleus of vagus nerve. The latter provides the motor innervetion to the LES and contains the efferent neurons that can either increase or decrease LES tone by stimulation of inhibitory or excitatory motor neurons in the myenteric plexus of the sphincter[1]. The excitatory myenteric neurons in LES are cholinergic in nature and act to stimulate muscarinic receptors on the smooth muscle[6]. Inhibitory motor neurons to LES are abundant and receive powerful

Table 1 Factors that regulate LES function

Factors that increase tLESRs	Factors that decrease tLESRs
Gastric distention	GABA agonists
Pharyngeal stimulation	Anticholinergics
High-fat meals	Opioid receptor agonists
CCK-A receptor agonists	Nitric oxide synthase inhibitors
	Endoscopic therapy
	Fundoplication

cholinergic nicotinic inputs from vagal efferents[6]. Although the vagal nerve innervates both excitatory and inhibitory myenteric motor neurons, vagal stimulation in experimental models invariably results in LES relaxation[7-9]. On the contrary, splachnic nerve stimulation relaxes the LES by activating adrenergic neurons, probably *via* nicotinic and non-nicotinic mechanisms of neural transmission, which in turn elicit beta-adrenergic inhibitory effects on the sphincter[10]. The inhibitory myenteric neurons innervating the LES are nitrergic in nature.

MECHANISMS OF GASTRO-ESOPHAGEAL REFLUX

The movement of gastric contents into the esophagus, or gastro-esophageal reflux, is due to a defective sphincter mechanism at the EGJ. An understanding of the two lower esophageal sphincters would lead one to expect weakness in either of the two to cause reflux. Indeed, some patients with reflux disease have a weak lower esophageal sphincter, some have a weak crural diaphragm, and some have both[2]. Reports suggest that it is possible to distinguish between two main mechanisms causing reflux: low basal sphincter pressure leading to free reflux, mostly occurring at night in the supine position, and increased frequency of transient lower esophageal sphincter relaxations with normal or increased resting LES pressure leading to reflux during the day in an upright position[11]. For example, in patients with mild-to-moderate (typically non-erosive) reflux disease, the pressures exerted by the lower esophageal sphincter[12] and the crural diaphragm[13] are normal. In such patients, the mechanism of reflux is due to transient spontaneous and inappropriate sphincter relaxations (tLESRs)[14].

A tLESR is a long period (lasting 10 to 60 s) of simultaneous relaxation of both the intrinsic distal esophageal sphincter and the crural diaphragm[2] representing a neural reflex that is mediated through the brain stem[2]. The efferent pathway for such relaxation is in the vagus nerve, and nitric oxide is the postganglionic neurotransmitter[15]. The mechanism of relaxation of the crural diaphragm is not known. Gastric distention and pharyngeal stimulation are two possible mechanisms by which the afferent stimulus that initiates transient relaxation of the LES may originate[16]. Gastric distention, upright and right lateral decubitus postures, and high-fat meals increase the frequency of such relaxation[16].

Diet

Patients with GERD are usually counseled on lifestyle changes that decrease the incidence of reflux, such as reducing fat in the diet. High dietary fat intake, particularly saturated fat, is associated with an increased risk of GERD symptoms and the likelihood of erosive esophagitis. These associations are independent of energy intake and therefore do not reflect a mere increase in total dietary intake. However, the effects are not completely independent of body mass index and are statistically significant only in overweight individuals. Other findings include a possible protective effect for high-fiber intake relative to GERD symptoms and a non-significant unfavorable trend for total energy intake[17].

An important role for dietary fat in causing temporary episodes of reflux is supported by studies of human volunteers that have shown increased frequency of tLESRs and increased esophageal acid exposure with high-fat consumption in both healthy volunteers[18,19] as well as patients with GERD[20,21]. Several food items have been associated with precipitating reflux symptoms in cross-sectional epidemiological surveys[22]. Previous case control studies reported a significant positive effect of fat intake on the rising rates of GERD and esophageal adenocarcinoma in the general population[23-25]. The fat content of the US food supply has increased 38% between 1909 and 1988[26]. These secular trends are consistent with the notion that high fat intake may be at least partially responsible for rising rates of esophageal adenocarcinoma, which were first observed in the late 1970s[17].

Sleep impairs esophageal acid clearance resulting in a prolongation of esophageal mucosal contact with acid[27]. Therefore avoiding meals two hours before lying down and sleeping with the head of the bed elevated prevents nocturnal acid reflux as nocturnal reflux *portends* a greater risk of erosive esophagitis and other GERD complications. Marked hyperglycemia has been shown to increase the frequency of tLESRs[28] and this may be a factor underlying the high rate of gastroesophageal reflux in patients with diabetes mellitus.

Pharmacologic control of LES

Since tLESRs represent the major mechanism responsible for episodic reflux, patients with GERD without mucosal disease or with mild erosive disease could potentially benefit from anti-tLESR therapy alone. Yet, this therapy could also be of value to treat some more severe forms of erosive esophagitis in combination with acid suppressants.

γ-GABA-b agonists: GABA is the major inhibitory neurotransmitter within the central nervous system, and GABA receptors are present at many sites within the central and peripheral nervous systems. GABA receptors are abundant pre-synaptically on vagal afferents in the dorsal medulla[29] and have been shown to inhibit neurotransmitter release in vagal nuclei[30]. Baclofen, a GABA analog, has recently been shown to be effective in reducing gastro-esophageal reflux[31,32]. Unlike acid suppressing agents, baclofen reduces the frequency of tLESRs and decreases the number of gastro-duodenal reflux episodes[33]. The drug has several advantages over other agents tested to date. It is available in an oral formulation and appears to have no adverse effects on other aspects of esophageal motor function, such as

basal LES pressure or acid clearance[34]. It also increases gastric pH in both GERD patients and controls even after repeated administrations. Since baclofen addresses a different factor in the pathophysiology of GERD, it can be used as add-on therapy in GERD patients with incomplete relief by acid suppression and/or in patients with more severe GERD[35]. However, baclofen has several limitations, including the need for frequent dosing due to its short half-life (3-4 h), and its limited absorption in the lower gastrointestinal tract, which precludes the development of a controlled-release formulation. The drug is nevertheless approved for treatment of spasticity, and its potential utility for the treatment of GERD has been demonstrated in clinical studies[36,37].

Recently, its active stereoisomer R-baclofen, has been incorporated into an experimental prodrug, XP19986. XP19986 overcomes the pharmacokinetic limitations of baclofen; it can be administered in a controlled-release formulation that allows for once- or twice-daily dosing. A recent Phase 1 clinical trial assessed the safety, tolerability and pharmacokinetics of XP19986 dosed once (QD) or twice (BID) daily in healthy adult volunteers. The trial was conducted sequentially in four separate cohorts of 12 subjects, three of whom received placebo per cohort. Following an up-titration period, the first cohort received 30 mg QD of XP19986 for seven days, followed by 30 mg BID for seven days and ending with a down-titration period. Subsequent cohorts received 60 mg and 90 mg QD and BID following a similar protocol. The final group of subjects received 120 mg QD only. Subjects were monitored for adverse events, and blood and urine were sampled to determine pharmacokinetic profiles and bioavailability. The trial demonstrated that repeated QD dosing of XP19986 resulted in sustained levels of R-baclofen in blood over 24-h, which reached steady-state within three days. Repeated BID dosing reduced the steady-state peak-to-trough ratio of R-baclofen by approximately 50% compared to QD dosing. Exposure to R-baclofen increased linearly with dose. This clinical trial also indicated that XP19986 was well tolerated. The most commonly reported adverse events were somnolence, dizziness and nausea that were generally mild in severity and generally increased in incidence compared to placebo with daily doses above 60 mg. In the 120 mg cohort, one subject experienced episodes of slurred speech and tremor, which are reported side effects of baclofen[38].

Cholecystokinin: The cholecystokinin A (CCK-A) receptor subtype is involved in the occurrence of tLESRs induced by gastric distension. Loxiglumide, a CCK-A antagonist, inhibits tLESRs and attenuates the fall in LES pressure following a meal, but has only modest effects on postprandial gastro-esophageal acid reflux[39].

Atropine: As mentioned earlier, the motor innervation to the LES and proximal gastric motor function is under cholinergic control[1]. Distension of the proximal stomach is a major stimulus for triggering tLESRs. Atropine, by acting centrally within the dorsal vagal complex, decreases the rate of tLESRs, despite the fact that it relaxes the proximal stomach and increases the degree of gastric distension under pressure-controlled conditions[40]. Anticholinergic agents are unlikely to have a therapeutic role because of the substantial deleterious effects on acid clearance[41].

Opioid receptor agonists: Opioid nerves have been demonstrated in the myenteric plexus of normal LES in humans[42] and opossum[43]. Morphine inhibits LES relaxation induced by swallowing and by balloon distension of the esophagus[44], thereby increasing residual LES pressure. On this basis, it seems likely that morphine might prevent reflux during tLESRs by rendering them incomplete. In one study[45] of patients with GERD, intravenous morphine inhibited tLESRs by 50%, and this effect was associated with a concomitant decrease in the rate of reflux episodes. Morphine also reduced the rate of tLESRs in normal subjects but by a lesser degree, and there was no decrease in the rate of reflux episodes, perhaps because the control rate was already relatively low. Interestingly, morphine did not appear to affect the residual pressure during tLESRs. The effect of morphine is completely blocked by naloxone, suggesting that it is mediated through μ-receptors. The effect of morphine is presumably central, because the peripherally active opiate loperamide does not influence the rate of tLESRs[46]. The effect of morphine on transient LES relaxations is dependent on the decrease in volume of the proximal stomach. Pharmacological interventions which decrease fundic volume should result in control of transient LES relaxation-mediated gastro-esophageal reflux[47]. Unwanted effects, such as addiction and constipation, are major barriers to the use of opioids[34].

Nitric oxide synthase inhibitors: Nitric oxide is the major post-ganglionic inhibitory neurotransmitter to the LES[34]. Nitrergic neurons are also present in the dorsal motor nucleus of the vagus, the source of pre-ganglionic motor neurons to the LES, and recent evidence suggests that there may be a novel nitrergic, pre-ganglionic, vagal pathway involved in LES relaxation[48]. It seems logical therefore, that blockade of nitric oxide synthesis might inhibit tLESRs. Indeed, in one study[49] the nitric oxide synthase inhibitor, NG-nitro-L-arginine-methyl ester (L-NAME), inhibited the rate of tLESRs, and this effect was reversed by L-arginine but not D-arginine. Other studies have found that, in normal humans, NG-monomethyl-L-arginine (L-NMMA) inhibited tLESRs induced by gastric distension by more than 75%[50], but reduced meal-induced tLESRs by only 25%[51]. Chronic administration of nitric oxide substrate, L-arginine, prolongs the postprandial increase in tLESRs[52].

The therapeutic potential of nitric oxide inhibition remains to be determined. At present, no orally effective agents exist. Inhibition of nitric oxide is associated with effects on motility in other areas of the gastrointestinal tract as well as in the cardiovascular system, urinary bladder, and respiratory tract. Unless specific and well-targeted antagonists of neuronal nitric oxide synthesis can be developed, unwanted side effects are likely to preclude this pharmacologic approach to the treatment of GERD[34].

Endoscopic methods that regulate LES function

Endoscopic endo-luminal anti-reflux approaches have

been recently introduced aiming to prevent acid reflux by construction of a functional or controlled barrier in the LES zone[53]. These endoscopic therapies offer the potential for significant symptomatic improvement while obviating many of the potential drawbacks associated with long-term medical therapy with acid suppressive or neutralizing medications, or the complications and side effects of anti-reflux surgery. These techniques include the delivery of radiofrequency energy to the EGJ (Stretta), injection of bulking agents (Enteryx), implantation of a bioprostheses into the LES (Gatekeeper), and suture plication of the proximal fundic folds (EndoCinch, NDOplicator)[54]. All these approaches are less invasive than antireflux surgery, and are performed in the outpatient setting. However, only limited data are available on their respective mechanisms of action[55]. Stretta increases the barrier between the stomach and the esophagus by either collagen deposition or disruption of vagally-mediated tLESRs[56]. The decrease in transient LES relaxation is presumably caused by either altering the mechanics of the cardia or interrupting afferent nerve transmission to the brainstem control mechanisms. Human and animal studies suggest that radiofrequency energy may decrease GERD symptoms[57] by decreasing the frequency of transient LES relaxations associated with reflux episodes, increasing the intra-gastric pressure needed to induce reflux, and hastening gastric emptying[58-61]. Another potential mechanism of the improved symptoms is decreased visceral sensitivity, as witnessed by the observation that the time needed to report symptoms during esophageal acid perfusion was significantly longer 6 mo after Stretta compared with baseline[62]. Although neurolysis with impairment of sensory afferent pathways is indeed a potential mechanism, other factors may include esophageal exposure to hydrogen ions, mucosal permeability, number and activation state of acid-sensitive nerve endings, and central processing of incoming sensory information. Radiofrequency energy delivery may influence several of these mechanisms as well as the compliance of the EGJ, increasing the resistance of the LES to a distending pressure[58] and decreasing the volume of refluxate during LES relaxations[57].

A variety of injectable agents have been studied for bulking the gastroesophageal junction, including Plexiglas microspheres, ethylene vinyl alcohol co-polymer (Enteryx), and hydrogel prosthesis (Gatekeeper). It is hypothesized that Enteryx leads to an increased barrier to reflux, whereas the hydrogel prosthesis may exert its effect by decreasing the aperture through which refluxate may flow, thus resulting in less proximal migration of the refluxate. Endoscopic plication is hypothesized to impede reflux by approximating tissue at or below the EGJ. The resulting effect of all these endoscopic techniques on LES pressure and 24-h intra-esophageal pH profiles is modest and complete normalization of acid exposure is uncommon. Nevertheless, all these techniques reduce GERD symptoms, improve quality of life, and decrease esophageal acid exposure and the need for anti-secretory medications[63,64].

Surgery

Laparoscopic fundoplication (LF) tightens the EGJ through a set of suture placations, resulting in significant increases in LES resting and post-deglutition relaxation pressures in both left lateral and upright positions and after gastric distention[65]. Post-fundoplication patients exhibit a diminished rate of tLESRs both at rest and during isobaric gastric distension compared with both normal controls and GERD subjects. Further, tLESRs in fundoplication patients are characterized by a higher residual pressure and a lower efficacy of facilitating gastric venting[66]. The reduction of tLESRs after a successful LF, is possibly due to disruption of the efferent vagal pathways to the LES. An adequate dissection of the EGJ alone prior to the actual fundoplication is sufficient to significantly disrupt the efferent pathway of this neural reflex, which may underscore the importance of performing a circumferential mobilization of the EGJ in all patients. Moreover, the wrap itself appears to further inhibit efferent neural impulses to the LES, thereby decreasing tLESRs[67].

Although the receptive field for triggering tLESRs is not completely clear[66], mechanoreceptors in the region of the gastric cardia are commonly implicated[16,68]. On the basis of studies in ferrets[69], two types of mechanoreceptors have been proposed: receptors in series with smooth muscle fibers, responding to wall tension variation, and receptors in parallel with smooth muscle fibers, responding to elongation of the gastric wall. Both types of receptors are activated during gastric distension[70,71]. However, after fundoplication the gastric cardia is situated within the fundic wrap, and has a reduced ability to stretch or elongate[72]. Thus an identical and constant distension stimulus likely leads to a diminished cardiac cross-sectional area compared with what would be observed if the same stimulus were applied in normal or GERD subjects. Reduced cross-sectional area would, in turn, result in decreased wall tension and elongation, thereby reducing the activation of both types of receptors potentially responsible for triggering tLESRs. Furthermore, because tLESR elicitation and gastric receptive relaxation are both mediated by the vagal nerve and since gastric accommodation is not affected by fundoplication[73], it is tempting to speculate that the vagal afferent field for triggering tLESRs is either independent of, or contained within a larger area responsible for elicitation of gastric receptive relaxation and that only the first is substantially reduced by fundoplication[66].

Postprandial epigastric fullness and bloating appear in 10%-80% of patients after Nissen fundoplication[74,75], and an inability to relieve this discomfort by belching is frequently reported[76]. Beyond the reduction in the number of tLESRs, a significant reduction of the percentage of tLESRs associated with a common cavity phenomenon is also found in fundoplication patients[77], and this potentially explains the frequently reported symptoms related to increased amounts of intestinal gas[66].

CONCLUSION

Given the increasing prevalence of GERD the efforts to regulate the EGJ and the LES continue along many fronts, pharmacologic, endoscopic, or surgical. Promising new ways to favorably alter the structure and function of the

EGJ and to reduce tLESRs may eventually replace acid suppression as the primary treatment of GERD or be used as adjunctive therapies.

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Venous thromboembolism with inflammatory bowel disease

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Abstract

Venous thrombosis and thromboembolism appear to be increased in patients with inflammatory bowel disease. Although several acquired and genetic risk factors are known, about half that develop a thromboembolic event have no identifiable risk factor. Control of the inflammatory process is thought to be the key factor in risk reduction for thrombotic events. Prophylactic use of anticoagulants is not universally recommended, but possible use should be reviewed in an individual patient after evaluation of the risks, such as hemorrhage, compared to potential benefits. Particular consideration should be given if there has been a prior thrombotic event, if hospitalization will require surgery, or if an underlying coagulation disorder is present.

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Key words: Venous thrombosis; Thromboembolism; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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INTRODUCTION

Some studies[1-5], but not all[6], have suggested that there is a higher incidence of venous thrombosis and thromboembolism in the clinical course of inflammatory bowel disease, especially in hospitalized patients[5]. In a population-based study[3], the risk was estimated to be about three-fold. Moreover, thromboembolism appears to develop with inflammatory bowel disease at a younger age[7]. Finally, thrombosis has also been reported in children with inflam-matory bowel disease[8].

ACQUIRED RISK FACTORS

Several risk factors have been hypothesized to contribute to development of thrombosis with inflammatory bowel disease. Some include the inflammatory process per se (with associated reactive thrombocytosis)[9], prolonged immobilization, surgical treatment, fluid depletion, central venous catheters (often for nutritional support), hyperhomocyteinemia, vitamin deficiencies, smoking and use of oral contraceptives[10]. Although hemostatic changes are frequent in inflammatory bowel disease patients, the precise reasons for thromboembolism during the course of inflammatory bowel disease are not known. Importantly, approximately half of the patients with inflammatory bowel disease that develop a thromboembolic event have no identifiable risk factor[11].

A number of specific coagulation abnormalities have been recorded in patients with inflammatory bowel disease that may lead to a thromboembolic event. Increased levels of factors V, Ⅶ and Ⅷ, lipoprotein(a) and fibrinogen, all risk factors for thrombosis, may occur. In addition, a reduction in natural anticoagulant factors including antithrombin Ⅲ, protein C and protein S may develop[10]. Homocysteinemia, another prothrombotic risk factor, may result from reduced folate, vitamin B6 or vitamin B12 levels. All of these vitamins may become deficient in patients with inflammatory bowel disease because of drug interference, impaired intake or malabsorption. To date, however, no consistent relationship with homocysteinemia and development of thromboembolism has been defined in patients with inflammatory bowel disease[12,13]. Finally, changes associated with subclinical activation of the coagulation cascade[14-16] along with diminished factor XⅢ have been reported[16,17].

GENETIC RISK FACTORS

Genetic factors may also play a role in patients with inflammatory bowel disease and have been reviewed elsewhere[18]. Some may be more significant during remission or less active phases of inflammation[19]. Factor V Leiden causes the activated form of factor V to be relatively resistant to degradation by activated protein C with

increased generation of thrombin. Factor V Leiden is often detected with venous thrombosis, but, to date, no difference between patients with inflammatory bowel disease and those without inflammatory bowel disease has been defined[18]. In addition, a genetic variation of the prothrombin gene, *G20210A*, may be associated with deep venous thrombosis, but no definite association between this gene mutation and inflammatory bowel disease has been detected[18]. Also, a mutation in the methylenetetrahydrofolate reductase gene, *C677T*, may cause hyperhomocysteinemia but no differences have been detected between inflammatory bowel disease patients with thrombosis compared to controls with thrombosis[18]. Finally, an inherited Val34Leu factor XIII polymorphism has been found to be similar in inflammatory bowel disease compared to the general population[20,21].

MANAGEMENT OF THROMBOTIC RISK AND THROMBOSIS

The key to reduction of risk for thombotic events in inflammatory bowel disease patients appears to be the control of the inflammatory process *per se*. Also, some of the medications used to treat ulcerative colitis and Crohn's disease may directly inhibit platelet activation. These include 5-aminosalicylic acid (5-ASA)-containing medications (eg., mesalazine, olsalazine)[22], azathioprine and its metabolite, 6-mercaptopurine (6MP)[23] and infliximab[24]. Correction of altered nutrition and vitamin deficiencies is also important, especially if surgery and prolonged periods of immobilization are expected. Smoking should be discouraged and ongoing use of medications associated with increased thrombosis risk should be reviewed, specifically, oral contraceptives.

Use of prophylactic anticoagulation (eg., subcutaneous heparin) is not universally recommended, but should be considered on an individual patient basis after evaluation of the potential risks compared to the potential benefits of prophylaxis. In a New York series, the risk of death due to pulmonary embolism was recorded in a single patient after surgical treatment from among 1000 patients with Crohn's disease (0.1%)[25]. Such information must be balanced by the potential risk of heparin treatment in inflammatory bowel disease, including the risk of hemorrhage[26]. Certainly, a case can be made for prophylaxis if there has been a prior thrombotic or thromboembolic event, if the patient is hospitalized for surgery, or if an underlying inherited coagulation disorder is known to be present.

Heparin and drugs that specifically target platelets may play a role in the treatment of the inflammatory process per se owing to their anti-inflammatory properties[27,28], but their precise role needs further evaluation in clinical trials. If thrombosis does supervene during the course of inflammatory bowel disease, management should not be different from treatment of patients without inflammatory bowel disease. However, acquired risk factors should be minimized and exclusion of genetic defects, if tests are available, should be considered. Clearly, additional fundamental studies are needed to further elucidate the role of this inflammatory process and its mediators in the pathogenesis of thromboembolism followed by large multicenter clinical studies that might define the role of emerging treatments, including newer biologic therapies.

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TOPIC HIGHLIGHT

Pier Alberto Testoni, MD, Associate Professor, *Series Editor*

Recurrent acute pancreatitis: From the editor

This special issue of *World Journal of Gastroenterology* contains a number of articles focusing on acute recurrent pancreatitis, a clinical entity that still remains a complex diagnostic and therapeutic challenge in clinical practice. Recurrent bouts of pancreatitis mainly occur in a gland which shows a normal morphology of both parenchyma and pancreatico-biliary ductal system at the time of diagnosis, when standard investigation is used; however, the introduction of ultrasound endoscopy (EUS) in clinical practice allowed us to identify mild to moderate ductal and parenchymal alterations in a relevant part of these patients, suggesting a chronic process, observed either at the onset of the disease or during the follow-up period. These pancreatic abnormalities can either suggest the presence of an underlying chronic process which evolves over time with recurrent attacks of acute pancreatitis or can be the consequence of multiple, self-limiting acute inflammatory episodes that induce persistent lesions within the gland with time.

It is generally believed that, in about 70% of cases, a correct aetiological diagnosis is achieved by means of clinical history, laboratory tests, and standard imaging techniques, including CT scan, magnetic resonance cholangio-pancreatography (MRCP), and endoscopic retrograde cholangio-pancreatography (ERCP).

Another significant improvement in the knowledge of aetiological factors has been achieved by the introduction of sphincter of Oddi manometry, microscopic search for bile crystals in the collected bile, and testing for cystic fibrosis transmembrane conductance regulator-gene (CFTR) and other genetic (SPINK1, PRSS1) mutations. Sphincter of Oddi manometry and the search for bile crystals may improve the diagnostic yield in patients in whom both pancreatico-biliary junction and ductal system have a normal appearance. By these techniques, the major role played by the sphincter of Oddi dysfunction, either of the biliary or pancreatic segment, and bile sludge or microlithiasis has been revealed in the occurrence of idiopathic recurrent pancreatitis. However, manometric investigation of the sphincter of Oddi may fail to document some dysfunction in a progressively increased percentage of patients in type 2 and 3 dysfunction, respectively, when the need for definite findings is highest. The introduction of the Secretin test, done by either MRCP or EUS investigation, has been documented to provide a more detailed visualization of the pancreatic ductal system and indirect information about the sphincter of Oddi function, avoiding ERCP-related risks.

The aim of the present topic highlights is to provide a comprehensive overview of the current knowledge and unsettled issues on recurrent acute pancreatitis, including aetiological factors, diagnostic and therapeutic procedures, and clinical management.

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TOPIC HIGHLIGHT

Pier Alberto Testoni, MD, Associate Professor, *Series Editor*

Is acute recurrent pancreatitis a chronic disease?

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Abstract

Whether acute recurrent pancreatitis is a chronic disease is still debated and a consensus is not still reached as demonstrated by differences in the classification of acute recurrent pancreatitis. There is major evidence for considering alcoholic pancreatitis as a chronic disease *ab initio* while chronic pancreatitis lesions detectable in biliary acute recurrent pancreatitis (ARP) seem a casual association. Cystic fibrosis transmembrane conductance regulator (CFTR) gene mutation, hereditary and obstructive pancreatitis seem an acute disease that progress to chronic pancreatitis, likely as a consequence of the activation and proliferation of pancreatic stellate cells that produce and activate collagen and therefore fibrosis. From the diagnostic point of view, in patients with acute recurrent pancreatitis Endoscopic ultrasound (EUS) seems the more reliable technique for an accurate evaluation and follow-up of some ductal and parenchymal abnormalities suspected for early chronic pancreatitis.

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Key words: Classification of pancreatitis; Acute recurrent pancreatitis; Chronic pancreatitis; Alcoholic pancreatitis; Pancreatic stellate cells

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THE CLASSIFICATION PROBLEM

Acute recurrent pancreatitis (ARP) is defined as the presence of at least two documented episodes of pancreatitis in a patient without chronic pancreatitis (CP) at imaging. The classification, however, is still a debated and controversial issue. Is this a relapsing acute disease with *restitutio ad integrum* of the pancreatic gland when the cause is eliminated or does it progress to chronic disease when the cause is not removed? There is also debate about whether ARP is actually a chronic disease *ab initio*, regardless of whether the cause persists, so that recurrences of pancreatitis are the clinical expression of CP in an early phase.

The term ARP was reported in the first Marseille classification of pancreatitis[1] that strictly divided ARP from CP. ARP is also reported in the classification of TIGAR-Ot[2], so-called from the acronym of the major predisposing risk factors, but was eliminated in the revised classifications of Marseille[3] and Marseille-Rome[4] because of the difficulties of distinguishing between a relapse of acute pancreatitis (AP) and an exacerbation of CP. In the paper referring to the Marseille-Rome classification the majority of the authors commented that the progression of acute to chronic pancreatitis was extremely uncommon.

In contrast, when pancreatitis is interpreted as a "hydraulic" obstructive problem, as a consequence of ductal lesions secondary to periductal inflammatory infiltrates[5] or post-necrotic scars[6,7], ARP can develop into CP over time if the cause of the disease persists. According to these pathogenic hypotheses, backed by experimental findings, the duration and grade of the pancreatic ductal obstruction are pivotal factors for differentiating the types of pancreatitis: partial, temporary pancreatic ductal obstruction (e.g. biliary lithiasis in humans) can lead to AP, while partial but long-lasting obstruction (e.g. stenosis of Oddi's sphincter or the Wirsung duct) is a cause of CP. When the ductal obstruction is complete and long-lasting (e.g. neoprene injection after surgery) the consequence is pancreatic atrophy.

AP, ARP and CP, according to this concept of "progression", are therefore not different diseases but different stages of the same disease. While biliary lithiasis and sphincter of Oddi dysfunction are the most frequent causes of AP and its recurrences, alcohol, smoking, genes and necrosis can all facilitate the progression from ARP to CP. A recent review[8] concluded that probably each of these classification concepts contains part of the truth, each providing one piece of the puzzle named pancreatitis.

ALCOHOLIC PANCREATITIS

Does acute recurrent alcoholic pancreatitis really exist or develop in a pancreas already affected by chronic disease? This question is still much debated. There are two schools

of thought: those who sustain that the relapses are an expression of acute disease that evolves to a chronic condition only if repeated over time, and those who maintain that the relapses are the expression of a chronic disease from the very start.

The differences are due to a different reading of the same results, the first more focused on the clinical picture, the second on histological data. The histological evidence of both acute necrotic and chronic lesions in tissue samples from six patients operated for a first acute attack of necrotic pancreatitis seems to support the idea that acute alcoholic pancreatitis develops in a pancreas already suffering from CP[9]. The same group[10] found similar results in 42 patients with alcoholic pancreatitis operated within two years of the clinical onset of the disease, investigated for histological evidence of diffuse or focal acute necrotic pancreatitis. However, only 7% of the patients had necrotic areas in the pancreatic tissue though all had the typical alterations of CP, such as perilobular and intralobular fibrosis, dilated inter- and intralobular ducts and protein plugs within the dilated ducts surrounded by periductal fibrosis, as already described several years ago[1]. These recent histological data confirm the detection of chronic lesions, in addition to the acute changes, previously reported in 12 patients with acute alcoholic pancreatitis, five of them at the first acute episode[11]. The authors suggested that alcoholic pancreatitis begins as a chronic disease and that the idea that repeated episodes of acute necrotic alcoholic pancreatitis cause CP is purely speculative since it is not supported by histology.

Ammann *et al*[12] conducted a sequential histological study of the pancreas in patients with alcoholic pancreatitis and concluded that it does progress from AP to CP, as suggested first by Comfort[6] and later by Kloppel[7], according to the "necrosis-fibrosis sequence" theory. Further evidence that recurrent pancreatitis can result in CP seems to come from studies of hereditary pancreatitis[13,14]. Whitcomb defined "sentinel acute pancreatitis (SAPE)" as an episode of sufficient severity to trigger the series of events that can lead to CP and proposed that a SAPE must occur for the development of CP[13].

The idea that ARP develops into a chronic disease has come under fire because, for example, in Amman's clinico-morphological study[12] all the patients had mild or severe chronic histologic pancreatic lesions already at their first histologic evaluation. Not only, the necrosis-fibrosis hypothesis fails to explain why some alcoholic patients with recurrent pancreatitis seem to progress to CP despite little evidence of necrosis and consequent pancreatic scarring.

The identification and characterization of stellate cells in the pancreas[15] and their vital role in fibrogenesis has led to further progress in understanding the pathogenesis of fibrosis in AP and CP, especially when caused by alcohol. The pathogenesis of alcoholic pancreatic fibrosis may involve two pathways: (a) a necro-inflammatory pathway involving cytokine release and activation of pancreatic stellate cells; (b) a non-necro-inflammatory pathway involving direct activation of stellate cells by ethanol through its metabolism to acetaldehyde and the generation of oxidant stress[16].

BILIARY PANCREATITIS

Gallstones are the most frequent cause AP. Sometimes patients with biliary ARP have ERCP findings of CP although it is not clear whether the two disease are linked by a cause-effect relationship (lithiasis as an etiological factor for CP) or by a casual association. While there are no data on patients with biliary ARP, abnormalities of the main pancreatic duct have been reported in about half of patients undergoing ERCP for biliary lithiasis[17].

In a retrospective study[18], biliary lithiasis in itself was not an etiological factor for CP: the frequency of main pancreatic duct abnormalities suggestive of CP by ERCP (increased diameter, irregular profiles, stenosis, dilation of the secondary ducts, cysts) was not significantly different in patients with biliary lithiasis, 68.4% of them with bile duct stones, and controls.

"GENETIC" PANCREATITIS

CFTR gene mutations

Recent studies showed that the frequency of cystic fibrosis transmembrane conductance regulator gene (CFTR) mutations is underestimated and that these have an important etiological and pathogenic role in so-called "idiopathic" ARP[19]. The proportion of patients with misdiagnosed cystic fibrosis and pancreatitis is 2%-3%, which is up to 75 times higher than the percentage reported in the general population [20,21]. The incidence of a single CFTR gene mutation in patients with ARP is significantly lower than in idiopathic CP (7.4% *vs* 15.5%, $P = 0.007$). However, it is difficult to establish whether a single CFTR gene mutation is sufficient to cause the clinical onset of pancreatitis or whether it is only a predisposing factor that needs some other cause, such as biliary microlithiasis, sphincter of Oddi dysfunction or other genetic mutations (SPINK1 or PRSS1).

CP patients with a single CFTR gene mutation had a significantly longer history of recurrent episodes of AP before the diagnosis of CP than patients with CP with a normal CFTR gene (7.4 ± 5.8 *vs* 2.1 ± 2 years; $P < 0.0001$). The mean interval between the first and second episode of AP was three years (range 1-6)[20].

The natural history of pancreatitis associated with CFTR gene mutation seems to be characterized by the onset of AP that can recur, leading, over variable periods of time, to dilation of the main pancreatic duct and hence to a diagnosis of CP. The CFTR gene mutation seems to confirm the possibility of progression from AP to CP as in hereditary and obstructive pancreatitis, but in a longer time than usually observed for CP[20]. However, it remains difficult to decide whether ARP associated with CFTR gene mutation is truly an acute condition that evolves to a chronic disease and not already chronic when it starts.

Hereditary pancreatitis (HP)

Patients with (HP) develop repeated episodes of AP that are indistinguishable from pancreatitis due to other causes. In their follow-up, patients with HP often present typical clinical, morphological and functional findings of CP, similar to those of the classic forms of CP[13].

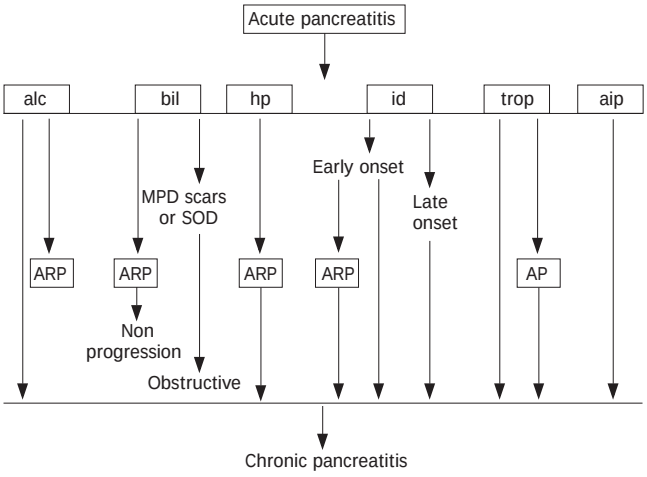


Figure 1 Progression from acute (AP) to chronic pancreatitis (CP). alc: Alcoholic; bil: Biliary; hp: Hereditary pancreatitis; id: Idiopathic pancreatitis; trop: Tropical; aip: Autoimmune; ARP: Acute recurrent pancreatitis; MPD: Main pancreatic duct; SOD: Sphincter of Oddi disfunction. (From ref. 23, with permission).

Recurrent attacks of mild or severe AP are more frequent in patients with the N9I mutation[22] and are associated with disabling pain, especially during the first years of the disease. Whitcomb thinks that the necrosis-fibrosis sequence hypothesis has some limitations in explaining why some patients with HP develop CP without significant pancreatic necrosis. He suggested that ARP might progress to CP when cytokines and chemokines are released from acinar cells during the acute episode and stimulate macrophages, with consequent infiltration, differentiation and/or proliferation of pancreatic stellate cells that produce collagen and therefore drive fibrosis. This might help explain the progression from ARP to CP as outlined by Uomo[23] (Figure 1).

IDIOPATHIC PANCREATITIS

An etiological diagnosis can be established in about two thirds of patients with ARP. No immediate cause is found in the remainder and ARP is considered "idiopathic". However, in some of these patients Endoscopic ultrasound (EUS) investigations have identified some ductal and parenchymal abnormalities suggesting a diagnosis of CP. Most of these patients had fewer than four EUS diagnostic criteria for CP[24] so that only those with pre-study high-risk conditions could be diagnosed as having CP. The recurrences of pancreatitis in these patients are quite likely the expression of a chronic disease *ab initio*. The frequency of the diagnosis of CP in patients with ARP, on the basis of EUS criteria, ranges from 10% to 30%[25,26]. Prospective randomized studies on ARP patients and controls, matched for sex and age, are therefore still needed to further verify the value of these criteria for this diagnosis.

In conclusion, the classification of ARP is still open to discussion. When CP is present, the diagnosis can usually be established on average almost five years after the second episode of AP. Episodes of pancreatic necrosis and gene mutations are suspected risk factors for the progression from ARP to CP, which may explain why, when genetic tests are available, the rate of ARP patients with a diagnosis of CP is higher.

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TOPIC HIGHLIGHT

Pier Alberto Testoni, MD, Associate Professor, *Series Editor*

Acute recurrent pancreatitis: An autoimmune disease?

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Abstract

In this review article, we will briefly describe the main characteristics of autoimmune pancreatitis and then we will concentrate on our aim, namely, evaluating the clinical characteristics of patients having recurrence of pain from the disease. In fact, the open question is to evaluate the possible presence of autoimmune pancreatitis in patients with an undefined etiology of acute pancreatitis and for this reason we carried out a search in the literature in order to explore this issue. In cases of recurrent attacks of pain in patients with "idiopathic" pancreatitis, we need to keep in mind the possibility that our patients may have autoimmune pancreatitis. Even though the frequency of this disease seems to be quite low, we believe that in the future, by increasing our knowledge on the subject, we will be able to diagnose an ever-increasing number of patients having acute recurrence of pain from autoimmune pancreatitis.

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Key words: Pancreatitis; Autoimmune pancreatitis; Diagnosis; Therapy; Outcome

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INTRODUCTION

In 1961, Sarles *et al*[1] reported the case of a non drinker patient suffering from pancreatitis associated with hypergammaglobulinemia. The authors hypothesized that the disease in this patient was an autonomous pancreatic disease of autoimmune origin. After this report, other authors around the world reported similar cases and they named the disease in several manners: chronic pancreatitis with diffuse narrowing of the pancreatic duct, primary inflammatory pancreatitis, non-alcoholic duct destructive chronic pancreatitis, lymphoplasmacytic sclerosing pancreatitis, granulomatous pancreatitis, idiopathic tumefactive chronic pancreatitis, and sclerosing pancreatocholangitis[2,3]. In 1995, Yoshida[4] suggested the term "autoimmune pancreatitis" for this disease and, therefore, this term has become largely accepted for pancreatic disease of an autoimmune origin. In the last 10 years, there have been an increasing number of cases reported in Japan and Europe[5]. In this review article, we will briefly describe the main characteristics of autoimmune pancreatitis and then we will concentrate on our aim, namely, evaluating the clinical characteristics of patients having recurrence of pain from the disease.

Incidence

At present, the exact incidence of the disease is not known. The only available data are those reported in Japan and in Italy. In these two countries, the estimated incidence of autoimmune pancreatitis is quite similar, 4.6% and 6.0% in Japan and in Italy, respectively[5] and we are awaiting data from the United States as well as from other countries in order to define the real incidence of the disease around the world. Autoimmune pancreatitis seems to have a preference for the male gender; in fact, about 80% of the cases described are males[5]. However, a geographic variation may be observed because, in Italy, the male: female ratio is 1:1. At diagnosis, the patients were more than 55 years of age[5]. Diabetes mellitus is present in about half of the patients[5].

Pathogenesis

From a pathological point of view, the disease is characterized by diffuse or focal pancreatic swelling with a narrowing of the pancreatic duct and/or common bile duct and the histological hallmark of this type of pancreatitis is lymphoplasmacytic infiltration, especially concentrated on the pancreatic ducts[6-8]. Some authors have defined autoimmune pancreatitis[9] as the simultaneous involvement of the pancreas, the salivary glands and the liver (primary biliary cirrhosis) by means of an immune-mediated inflammatory process. Thus, the still open question is the differentiation of autoimmune pancreatitis as a primary or a secondary disease based on the absence or presence of other autoimmune diseases.

Clinical aspects

From a clinical point of view, patients with autoimmune

pancreatitis rarely complain about the typical severe abdominal pain of pancreatitis and are usually hospitalized for painless jaundice[10]; other symptoms of autoimmune pancreatitis include non-specific mild abdominal pain and weight loss. The diagnosis is sometimes quite intriguing because the disease may be mistaken for pancreatic cancer[11].

Laboratory data

Laboratory analysis is undergoing continuous evolution. Serum amylase and lipase may often be normal or a mild elevation of the serum pancreatic enzymes may be observed, and in only a few cases is there a marked elevation of these pancreatic damage markers[12]. Hypergammaglobulinemia and IgG serum increase have been reported in percentages ranging from 37% to 76%[13,14]. Japanese authors have claimed that elevated serum levels of IgG4, a subtype of IgG, are a biochemical hallmark of autoimmune pancreatitis[15]; however, other authors have recently questioned the specificity of the IgG4 because elevated IgG4 levels are present in patients suffering from pancreatic carcinoma and other types of chronic pancreatitis[5].

Non-specific autoantibodies, such as antinuclear antibodies, antimitochrondial antibodies and so on have a low sensitivity in diagnosing autoimmune pancreatitis; the detection rate of specific antibodies such as antilactoferrin antibodies and anticarbonic anhydrase Ⅱ antibodies have not been widely assessed in clinical setting because they require a special laboratory for their measurement which is available to only a low number of clinicians. A number of groups have tried to find other laboratory indicators of autoimmune pancreatitis and evaluation of the alleles of major histocompatibility complex genes seems to be a promising tool for identifying patients susceptible to autoimmune pancreatitis. One report mentioned that DRB1*0405 and DQB1*0401 are significantly more frequent in patients with autoimmune pancreatitis when compared to chronic calcifying pancreatitis[16]. At the present time, however, further studies are required to evaluate the value of each laboratory indicator and to find a more reliable one.

Imaging evaluation

Imaging evaluation is essential in the diagnosis of autoimmune pancreatitis[17]. Ultrasound is often the first imaging technique to be utilized in a patient with obstructive jaundice or with upper abdominal pain and a hypoehoic diffuse swelling in the pancreas (sausage-like appearance), or a focal swelling of the pancreas simulating a neoplastic lesion can be observed as well as a dilation of the extrapancreatic bile duct, secondary to an involvement of its intrapancreatic portion. Contrast-enhanced ultrasonography can successfully visualize fine vessels in pancreatic lesions and may play a pivotal role in the depiction and differential diagnosis of pancreatic tumors. In particular, some Authors have analyzed the enhancement of focal pancreatic lesions and it has been shown that, while most of the inflammatory pancreatic masses show a pattern of enhancement similar to the normal pancreatic gland ("isovascular"), a focal pancreatic tumor is hypovascular to the surrounding normal parenchyma At both computed tomography and magnetic resonance imaging, a focal or diffuse swelling of the pancreatic gland can be observed. Dynamic imaging at computed tomography or magnetic resonance imaging shows a delayed enhancement of the segments of the gland, which are involved. In some cases, minimal peripancreatic stranding suggesting inflammation can be seen. Moreover, a capsule-like smooth rim can be observed which is hypodense on computed tomography and hypointense on T2 weighted images, showing delayed enhancement on dynamic imaging. This is thought to correspond to an inflammatory process involving peripancreatic tissues and appears to be a characteristic finding of autoimmune pancreatitis. Pancreatic calcifications are rarely seen in autoimmune pancreatitis. Involvement of the main pancreatic duct and the biliary duct is well-described in the literature. Endoscopic retrograde cholangiopancreatographic criteria for the diagnosis of autoimmune pancreatitis include diffuse irregular narrowing of the main pancreatic duct and abnormalities which normalized after steroid therapy. The same alterations can be observed at MR cholangiopancreatography. The invasion of vessels, vascular encasement, mass effect and fluid collections are absent in autoimmune pancreatitis.

Diagnostic criteria

The diagnostic criteria currently used for autoimmune pancreatitis are those proposed by the Japan Pancreas Society[18] and are reported in Table 1; interestingly, the criteria do not include symptoms or common laboratory findings as they are not specific to autoimmune pancreatitis[12,19]. Italian criteria include some differences with respect to the Japanese diagnostic criteria (Table 2)[5] such as the association with other autoimmune diseases and the response of the disease to steroid treatment. Korean researchers utilize a third classification which takes into account the Japanese and the Italian diagnostic criteria (Table 3)[20]. Furthermore, a fourth classification system has been proposed from the same Korean researchers (Table 4)[20]. It seems that there is a need for a classification system for such a rare disease; therefore, an international consensus statement releasing widely accepted guidelines for autoimmune pancreatitis would be welcome.

Open questions

The open question is to evaluate the possible presence of autoimmune pancreatitis in patients with an undefined etiology; in fact, a recent study has reported that clinical or biochemical autoimmune stigmata are present in 40% of patients with idiopathic chronic pancreatitis and, therefore, autoimmune mechanisms may be frequent in idiopathic pancreatitis[21]. We also need to know the possible cause of failure of steroid therapy in some patients; finally, we need to evaluate the reason why some patients experience more attacks of pain in a disease characterized by a painless course. We carried out a search in the literature in order to explore this latter issue.

Table 1 Diagnostic criteria for autoimmune pancreatitis released by the Japan Pancreas Society

Criteria	Definition
Ⅰ Imaging criterion	Diffuse narrowing of the main pancreatic duct with an irregular wall (more than 1/3 length of the entire pancreas) and enlargement of the pancreas
Ⅱ Laboratory criterion	Abnormally elevated levels of serum gammaglobulin and/or IgG, or the presence of autoantibodies
Ⅲ Histopathologic criterion	Marked lymphoplasmacytic infiltration and dense fibrosis

For diagnosis, criterion Ⅰ must be present, together with criterion Ⅱ and/ or Ⅲ.

Table 2 Italian diagnostic criteria for autoimmune pancreatitis

Criteria	Definition
Criterion Ⅰ	Histology and cytology
Criterion Ⅱ	Association with other postulated autoimmune disease
Criterion Ⅲ	Response to steroid therapy

One or more criteria must be present in order to diagnose autoimmune pancreatitis.

DEFINITION OF RECURRENT PANCREATITIS

For the aim of this study, we defined acute recurrent pancreatitis as the presence of an attack of pancreatic pain in patients with proven autoimmune pancreatitis.

SEARCH LITERATURE PROCEDURE

On August 1st 2006, we performed a search using three different data bases (MEDLINE, Web of Science and Scopus) in order to find the data existing in the literature on the presence of recurrent pancreatitis in patients with autoimmune pancreatitis. The terms "recurrent pancreatitis" and "autoimmune pancreatitis" were searched as topics for MEDLINE, as Topic terms (TS) for Web of Science (WoS) and as article titles, abstracts and keywords containing the two terms for Scopus. One-hundred and twenty-six papers on MEDLINE, 15 on WoS, and 14 on Scopus were found. Only papers regarding studies carried out on humans, in English and in full text were considered. Fourteen duplicate papers were found among the various databases; 10 of these were present simultaneously in MEDLINE, WoS and Scopus, three were present in both Scopus and WoS while the remaining one was present in both MEDLINE and Scopus. One-hundred and thirty-one papers were found. Of these, 24 papers were excluded because they were review articles[4,12,22-43] and one was an editorial letter to the editor not reporting original data[3]. The remaining 106 original papers were considered for the purpose of this study. Of these 106 original papers, 18 were excluded because they reported data not useful for the aim of this study[44-61]. Therefore, 88 studies satisfied the aim of our study; however, 79 of these papers do not report data on the recurrence of acute pancreatitis in the follow-up of patients with autoimmune pancreatitis[6,14,62-138]. Therefore, 9 papers were evaluated for the purpose of this study[13,139-146].

Table 3 Korean diagnostic criteria for autoimmune pancreatitis released by the Asian Medical Center

Criteria	Definition
Criterion Ⅰ	Pancreatic imaging (essential): (1) CT: Diffuse enlargement (swelling) of pancreas and (2) ERCP: Diffuse or segmental irregular narrowing of main pancreatic duct
Criterion Ⅱ	Laboratory findings: (1) elevated levels of IgG and/or IgG4 or (2) detected autoantibodies
Criterion Ⅲ	Histopathologic findings: Fibrosis and lymphoplasmacytic infiltration
Criterion Ⅳ	Response to steroids

Definite diagnosis: Criterion Ⅰ and any of criteria Ⅱ-Ⅳ.

Table 4 A proposal of revised Korean diagnostic criteria for autoimmune pancreatitis

Criteria	Definition
Criterion Ⅰ	Pancreatic imaging (essential): (1) CT: Diffuse enlargement (swelling) of pancreas and (2) ERCP: Diffuse or segmental irregular narrowing of main pancreatic duct
Criterion Ⅱ	Laboratory findings: (1) elevated levels of IgG and/or IgG4 or (2) detected autoantibodies
Criterion Ⅲ	Histopathologic findings Fibrosis and lymphoplasmocytic infiltration
Criterion Ⅳ	Association with other postulated autoimmune disease

Definite diagnosis: Ⅰ + Ⅱ + Ⅲ + Ⅳ or Ⅰ + Ⅱ + Ⅲ or Ⅰ + Ⅱ or Ⅰ + Ⅲ; Probable diagnosis: Ⅰ + Ⅳ (Rediagnosed as "definite" if "response to steroids" is present); Possible diagnosis: Ⅰ (Rediagnosed as "definite" if "response to steroids" is present).

RESULTS

A total of 15 patients with recurrent episodes of acute pancreatitis during the course of autoimmune pancreatitis are reported with a clinical follow-up ranging from 5 to 276 mo and their data are shown in Tables 5 and 6.

Taking into consideration only the studies of Hardacre[142], Weber[145] and Zamboni[146] which reported a high number of patients, the frequency of autoimmune pancreatitis patients with recurrent abdominal pain was 2.7% (1/37), 3.2% (1/31) and 4.5% (1/22) in the three above-mentioned studies with a mean frequency of 3.3%.

Considering all the cases reported with recurrence of pain, most of the patients were under 50 years of age and the ratio male: female was near to one. The major part of the patients (5/8, 62.5%) had more than one recurrence; some experienced more than 4 recurrences of pain in the follow-up period and most of them were operated on. In the study of Wayne *et al*[144], two of the five patients with recurrent pancreatitis underwent pancreatic surgery 2 and

Table 5 Clinical and morphological data of autoimmune pancreatitis patients with recurrent pancreatitis

Study	Type of study	Patients studied	Associated disease	Median FU (mo)	FU range (mo)	Criteria adopted	Imaging alterations	IgG elevation	IgG4 elevation	Antibodies present
Borum[140]	CR	2	SLE		132-253	Histology	Yes	NR	NR	Yes
Wakabayashi[13]	R	7	5 U		28-75	JPS	Yes	5/7	NR	1/8
Hardacre[142]	R	37	1 SS, 2 UC	33	NR	Histology	NR	NR	NR	NR
Weber[145]	P	31	1 BP + LD + F, 1 IN, 1 Pa, 1 RA, 1 SS+GD+AI, 1 UC+GS+G	38	0-72	Histology	Yes	NR	NR	NR
Fernandez-del Castillo[141]	CR	1	None	5		Histology	Yes	NR	NR	NR
Zamboni[146]	R	53	2 SS, 1 SS + T, 1 SS + RF, 2 CD, 1 CD + RA, 3 UC, 4 U	36[1]	6-276[1]	Histology	NR	NR	NR	NR
Tanvetyanon[143]	CR	1	MS	14		JPS	Yes	NR	No	No
Wayne[144]	R	6	None	60	48-240	Histology	3/6	NR	NR	1/7
Abisi[139]	CR	1	IN, SS, LA	NR	NR	Histology	Yes	Yes	NR	Yes

FU: Follow-up; CR: Case report; R: Retrospective; P: Prospective; JPS: Japanese Pancreatic Society criteria for autoimmune pancreatitis; NR: Not reported; SLE: Systemic lupus erythematosus; SS: Sjogren syndrome; UC: Ulcerative colitis; BP: Bell's palsy; LD: Lyme disease; F: Fibromylagia; IN: Interstitial nephritis; Pa: Parotiditis; RA: Rheumatoid arthritis; GA: Grave's disease; AI: Adrenal insufficiency; GS: Granulomatous sialoadenitis; G: Goiter; T: Thyroiditis; RF: retroperitoneal fibrosis; CD: Crohn's disease; LA: Lupus anticoagulant; MS: Myleodysplastic sindrome: U: Unspecified. [1]The follow-up period has been reported for only 22 patients.

Tabella 6 Clinical characteristic, medical/surgical treatment and complications of autoimmune pancreatitis patients with recurrent pancreatitis

Study	No. patients with acute recurrent pancreatitis	Age at diagnosis (yr)	Sex	Localization	No. of recurrences	Surgery	No. of operation	Type of surgery	Steroid therapy	Improvement with steroids	Pancreatic complication
Borum[140]	1	34	Female	Tail	2	Yes	1	DP	Yes	No	Calcifications Pseudocyst
Wakabayashi[13]	3	NR	NR	NR	1	No			NR		NR
Hardacre[142]	1	NR	NR	NR	NR	Yes	1	NR	No		NR
Weber[145]	1	NR	NR	NR	1	No			No		NR
Fernandez-del Castillo[141]	1	36	Male	Head	> 2	Yes	1	PD	NR		NR
Zamboni[146]	1	49	Male	Head	1	Yes	1	PD	No		No
Tanvetyanon[143]	1	44	Male	Diffuse	> 10	No			Yes	Yes	Pseudocysts
Wayne[144]	5	30 (25-39)[1]	5 females	3 tail, 2 diffuse	2-> 10	Yes	1 (1-3)*	4 DP, 2 PD, 1 PP, 1 TP	1/5	No	1 pseudocyst, 1 calcifications
Abisi[139]	1	78	Male	Diffuse	4	Yes	2	1 DP, 1 CJ+G	Yes	Yes	Calcifications Pseudocyst

DP: Distal pancreatectomy; PD: Pancreaticoduodenectomy; PP: Puestow procedure; TP: Total pancreatectomy; CJ: Choledochojejunostomy; G: Gastroenterostomy; NR: Not reported. [1]median and (range).

3 times. Information about the presence of pancreatic complications was reported in nine patients; pseudocysts and pancreatic calcifications developed in more than 50% of the cases. It is worth noting that, in most of the patients studied, the diagnosis was only histologically proven and the Japanese criteria for diagnosis acute pancreatitis were carried out in only two studies[13,143]. Therefore, imaging techniques in patients having autoimmune pancreatitis with a recurrent attack of pain seem to have a limited role in preoperatively diagnosing the disease; furthermore, the serological indices of autoimmunity were carried out in only a limited number of patients. Regarding medical therapy, steroids had no beneficial effects on 2 of the 4 patients on whom the treatment was carried out.

PRACTICAL CONSIDERATIONS

The frequency of recurrent pancreatitis in patients with autoimmune pancreatitis seems to be very low. In fact, if we considered that the frequency of autoimmune pancreatitis is of about 5%, in these patients, the frequency of recurrent attacks of pain is of about 3%. Furthermore, in these patients, steroids seem to be effective only in an half of them and the number of pancreatic operations is quite high in. We probably need to utilize more extensively the information given by imaging techniques and serological indices compatible with the disease in order to decrease a false diagnosis in autoimmune pancreatitis patients with recurrent attacks of pain. However, in this regard we need to remember that, in recent years, the accuracy of IgG4 in diagnosing the autoimmune forms

of pancreatitis seems to be lower than that reported in the past[5]. Furthermore, we need a world consensus on the diagnostic work-up of autoimmune pancreatitis because the criteria utilized until now seem to be inadequate[5,91]. We also need a well-executed international study in order to obtain more information regarding the recurrent pain in patients with autoimmune pancreatitis in order to increase our knowledge of the risk factors of recurrence, diagnosis, and therapy.

CONCLUSION

In cases of recurrent attacks of pain in patients with "idiopathic" chronic pancreatitis, we need to keep in mind the possibility that our patients may have autoimmune pancreatitis. Even though the frequency of this disease seems to be quite low, we believe that in the future, by increasing our knowledge on the subject, we will be able to diagnose an ever-increasing number of patients having acute recurrence of pain from autoimmune pancreatitis. The possibility of finding these diagnostic possibilities and treatment for this disease is still a challenge.

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TOPIC HIGHLIGHT

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Diagnostic approach to patients with acute idiopathic and recurrent pancreatitis, what should be done?

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Abstract

Acute recurrent pancreatitis (ARP) is a common clinical condition that may be difficult to diagnose. Endoscopic ultrasound (EUS) is proposed to be a safe first line test of choice in the majority of patients. When interventions are needed to remove biliary stones, evaluate sphincter of Oddi or pancreas divisum, endoscopic retrograde cholangiopancreatography (ERCP) is recommended. Magnetic resonance cholangiopancreatography (MRCP) can be a suitable alternative from a diagnostic standpoint although may not be widely available. Finally, genetic testing is increasingly used to detect certain mutations that are associated with this diagnosis.

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Key words: Acute recurrent pancreatitis; Idiopathic pancreatitis; Endoscopic ultrasound; Endoscopic retrograde cholangiopancreatography; Magnetic resonance cholangiopancreatography

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INTRODUCTION

Acute recurrent pancreatitis (ARP) is a common, potentially life-threatening condition that requires a combination of sophisticated medical and interventional skills to diagnose, determine the etiology and treat. After an initial episode of pancreatitis studies suggested that 30% of the patients will become experience recurrence[1]. he etiology of ARP has been evaluated in a number of classic studies with alcohol and gallstones were found to be the major contributors[1-3], accounting for 70% of the cases[4].

According to Tandon *et al*[5], "idiopathic acute pancreatitis" was defined as pancreatitis with no etiology established after initial laboratory (including lipid and calcium level) and imaging (either transabdominal ultrasound and/or CT scan) tests. However, there is no consensus on the duration of an "acute attack", as pancreatic parenchymal changes such as edema and fluid collection may persist after the complete normalization of pancreatic enzymes and resolution of patient's symptoms. The definition is less specific if the interval between the acute attacks is short, where inflammation and edema may persist. For the purposes of this discussion, we will define acute pancreatitis according the Atlanta Symposium[6] as an acute inflammatory process of the pancreas, usually associated with severe pain in the upper abdomen with a 3-fold rise in amylase and lipase. ARP is defined as at least 2 episodes of acute pancreatitis with complete or near complete resolution of symptoms and signs of pancreatitis between episodes.

DIAGNOSTIC EVALUATION

ARP remains a diagnostic dilemma. It is estimated that 10% of a single pancreatitis cases and up to 30% of recurrent pancreatitis remain undiagnosed after history, laboratory testing, and routine imaging[7,8].

Initial laboratory testing should include serum calcium and triglycerides. Although an uncommon complication, hypercalcemia of any cause can lead to acute pancreatitis[9,10]. Suggested mechanisms include deposition of calcium in the pancreatic duct and calcium activation of trypsinogen within the pancreatic parenchyma[10]. Serum triglyceride concentrations above 1000 mg/dL (11 mmol/L) can precipitate attacks of acute pancreatitis[11], although the mechanism of inflammation in this case remains unclear.

The combination of bile crystal analysis and endoscopic retrograde cholangiopancreatography (ERCP) with or without sphincter of Oddi manometry (SOM) has led to identifying the underlying etiology up to 76% of cases of otherwise unexplained acute pancreatitis[1,8]. The advent of EUS and the rapid evolution of various imaging modalities have provided a less invasive highly accuracy alternative for studying the pancreas and the biliary tree. In this review, we will focus on the diagnostic approach to patients with ARP including medical testing, imaging and endoscopy.

EUS OR ERCP?

ERCP has been used for decades in the evaluation of idiopathic recurrent pancreatitis. The role of ERCP emerged prominently in the diagnosis of underlying cholelithiasis[12,13], papillary stenosis[1], pancreas divisum[14] and sphincter of Oddi dysfunction[1]. The major limitation of ERCP is the risk of inducing acute pancreatitis, which may be as high as 20% in patients being evaluated for SOD and is 5%-6% in the general population[15]. Over the past 10 years, EUS has replaced ERCP for most diagnostic evaluations due to its high accuracy (at least for bile duct stones, tumors, pancreas divisum and cysts) and lower risk of complications[16]. The major limitation of EUS is the inability to clearly diagnose SOD.

Coyle *et al*[13] studied the diagnostic utility of ERCP with SOM, bile analysis and EUS in patients with acute and ARP. A diagnosis was found in the majority of the cases (80%), where SOD was the most common finding (31%), while pancreatic divisum was found in 20% of patients. EUS was very useful in the diagnosis of biliary diseases and detected all tumors. Endoscopic ultrasound (EUS) was able to identify 9 patients with chronic pancreatitis, which ERCP did not identify. However, EUS was not able diagnose any patient with SOD.

In a similar study by Fossard *et al*[17] EUS identified the cause of acute pancreatitis in 77% of patients. EUS was abnormal EUS were compared with other investigations, EUS correctly identified the cause of the "idiopathic" pancreatitis in 155 (92%) of the patients, most commonly being biliary tract disease. EUS missed the diagnosis in 5 patients with biliary stones and one patient with intraductal papillary mucinous neoplasm (IPMT). The authors concluded that EUS is valuable in determining the cause of acute pancreatitis in patients initially considered to have idiopathic pancreatitis.

According to Tandon *et al*[5], EUS was successful in determining the etiology of idiopathic acute pancreatic in 21 out of 31 patients (68%). The diagnoses made by EUS were: chronic pancreatitis (45%), microlithiasis (16%), pancreas divisum (6.5%) and pancreatic carcinoma (3.2%), and was not diagnostic in 10 (32%) The mean follow up was 16 mo. Thirteen patients in this study had further recurrent pancreatitis or pancreatic symptoms and underwent further investigations. Only nine of these patients required ERCP and four patients had a change of their previous EUS diagnoses. Hence, 87% (27 of 31 patients) of the initial EUS diagnoses were *unchanged* at the end of follow up. Two patient groups were included in this study and provoked criticism: patients with significant alcohol consumption and patients with only single episode of pancreatitis.

A prospective study of 300 patients of single episode and recurrent pancreatitis by Yusoff *et al*[18] concluded that it is reasonable to perform EUS after the first episode of pancreatitis. Diagnostic yield of EUS was comparable between the 2 groups (single *vs* recurrent episodes). Patients with significant alcohol intake and with features of chronic pancreatitis were excluded from this study.

WHEN CAN EUS REPLACE ERCP?

The most likely area where EUS can replace ERCP, at least as the initial diagnostic test, is when biliary tract disease such as stones, are suspected. Prat *et al*[19] studied the value of early EUS to avoid unnecessary cannulation of the bile duct in patients with acute biliary pancreatitis. ERCP was only performed if biliary stones were seen by EUS. There were no false positive EUS results and no EUS related complications. Also, Napoleon and his colleagues[20] found that the negative predictive value of EUS for the diagnosis of CBD stones was 95.4%. Therefore, they concluded that EUS should effectively replace ERCP as the initial diagnostic test of choice.

Two other studies also examined the utility of EUS in idiopathic pancreatitis suspected to be of biliary origin. Norton and Alderson[21] found that pancreato-biliary causes of idiopathic acute pancreatitis can be established in at least 18 of 44 patients (41%) studied. Although this diagnostic yield is lower compared with 68% suggested by Tandon[5], patients with significant alcohol intake were excluded in the Norton study. In addition, Liu *et al*[22] found that EUS led to the correct diagnosis in 14 of 18 patients (78%) with idiopathic pancreatitis of choledocholithiasis and/or cholelithiasis. Although this study excluded patients with history of excess alcohol ingestion, it included patients with only one episode of pancreatitis. As for IPMT, EUS has a sensitivity of 86% and specificity of 99% in diagnosing IPMT[23].

Autoimmune pancreatitis (AIP) is a rare but increasingly diagnosed clinical entity[24,25], which can present as pancreatic or biliary strictures, acute recurrent pancreatitis or even pancreatic mass. Elevated serum levels of IgG4 provide a strong marker for the disease[26,27]. Serum IgG4 accounts for only 5 to 6 present of the total IgG4 in healthy subjects but is elevated in patients with AIP. A large study by Hamano *et al*[27] compared serum IgG4 levels in 20 patients with AIP with 20 healthy controls, and 154 patients with a variety of malignant and non-malignant pancreatic diseases. The median serum IgG4 concentration in patients with AIP was 663 mg/dL compared with 51 mg/dL in healthy controls. The serum IgG4 concentrations in the other group of patients were similar to those in healthy subjects with no values above 135 mg/dL. Using a cutoff of 135 mg/dL, the sensitivity and specificity of the serum IgG for distinguishing AIP from pancreatic cancer were 95 and 97 percent, respectively. Levels of IgG4 declined during treatment with corticosteroids. Classic endosonographic features include diffusely hypoechoic, enlarged pancreas together

with chronic inflammatory cells on aspirated cytologic specimens[28]. However, EUS fine needle aspiration (FNA) is usually insufficient to diagnose autoimmune pancreatitis due to the small sample size and lack of tissue architecture. In a study by Levy *et al*, five patients in whom the diagnosis was considered were evaluated. A mean of 2.8 EUS truecut biopsy (range 2-5) was obtained per patient[29]. Truecut biopsy specimens were diagnostic of AIP in two patients and were strongly suggestive but not diagnostic in two others. In one patient only nonspecific changes of chronic pancreatitis were seen on histology. EUS FNA (mean 4.3 passes, range 2-7 passes) was performed in four of five patients and failed to establish or suggest the diagnosis in any of these patients. Serum IgG4 and EUS should be considered first line tests in cases where AIP is suspected.

A major advantage of ERCP compared to EUS is the ability to perform manometry and, if needed, intervention if specific etiologies have been found in the same setting. For the common causes found in patients with unexplained pancreatitis, sphincterotomy (either biliary or pancreatic) or stent insertion could be performed. To overcome this disadvantage, Rocca *et al* recently presented a prototype oblique-viewing echoendoscope capable of performing EUS and ERCP in the same session[30]. Nineteen patients with acute abdominal pain associated with increased liver tests entered the study and underwent EUS. When biliary stones or sludge were found, bile duct cannulation and sphincterotomy were performed in the same session. Bile duct cannulation failed in only one patient and no procedure-related complications were observed. The authors concluded that this approach was accurate, safe and feasible for the treatment of common bile duct stones when needed. Further larger studies are needed to confirm these findings.

MRCP

MRCP represents a very useful radiographic tool for the assessment of the intra and extrahepatic biliary tree and pancreatic duct. In the recent years, the concurrent use of secretin makes MRCP an attractive first line test for ARP to assess for structural underlying etiologies. Until recently, sphincter of Oddi evaluation remained almost completely dependent on endoscopic approach. However, a study by Mariani *et al* compared secretin MRCP and pancreatic sphincter of Oddi manometry for evaluation of sphincter of Oddi function in patients with idiopathic ARP[31]. Eighteen patients and a similar number of controls underwent both tests and data from 15 patients was available for analysis. Secretin MRCP and sphincter of Oddi manometry were concordant in 13/15 patients (86.7%); positive and negative diagnoses for sphincter of Oddi dysfunction agreed in, respectively, 81.8% and 100% (kappa value 0.706).

Another study by Khalid *et al*[32] evaluated pancreatic duct outflow obstruction in cases of idiopathic ARP. Ten patients with idiopathic acute recurrent pancreatitis underwent secretin-stimulated magnetic resonance pancreatography with subsequent endoscopic retrograde pancreatogram with or without manometry. It was found that secretin stimulated magnetic resonance pancreatography provides high quality pancreatic duct images and has high specificity for diagnosing pancreatic duct outflow obstruction using manometric and clinical criteria.

GENETIC TESTING

Cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations were noted to be associated with cystic fibrosis (CF)-related conditions, including idiopathic recurrent pancreatitis in otherwise non-symptomatic patients. A prospective study by Bishop *et al* included idiopathic ARP, healthy controls, CF heterozygotes, and CF patients (pancreatic insufficient or sufficient) for evidence of CFTR gene mutations. At least one CFTR mutation or variant was carried in 6 of 16 patients (38%) with idiopathic ARP but in only 11 of the 50 controls (22%, $P = 0.005$). Identified mutations were rare and are not usually identifiable with the routine screening test. While genetic testing may carry more significance in the diagnostic work up of chronic pancreatitis, acute pancreatitis can be viewed as an event that leads to the process of chronic pancreatitis. The role of other mutations involving cationic trypsinogen (PRSS1) and in serine protease inhibitor Kazal type 1 (SPINK1) genes seen in idiopathic chronic pancreatitis is less established in ARP and further studies are required[33,34].

CONCLUSION

Each of the above-described modalities to diagnose ARP has its pros and cons. Despite the fact that there are limitations in using EUS to investigate ARP, it remains a reasonably accurate and less invasive alternative to ERCP for patients with only one episode of pancreatitis. For certain cases of recurrent pancreatitis, besides being able to perform interventions, ERCP offers additional information about sphincter of Oddi function, and also in cases of pancreas divisum where the sensitivity and specificity of EUS needs further evaluation. In other cases where biliary stones or IPMT are suspected, EUS remains a less invasive and equally accurate test of choice. The risks and benefits of these procedures should be individually evaluated in each case. MRCP with secretin stimulation is a non-invasive, promising tool but requires advanced interpretation skills and may not be widely available. In a subset of patients where structural causes have been ruled out, genetic testing may be helpful in the appropriate clinical set-up.

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TOPIC HIGHLIGHT

Pier Alberto Testoni, MD, Associate Professor, *Series Editor*

Role of genetic disorders in acute recurrent pancreatitis

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Abstract

There was remarkable progress in the understanding of the role genetic risk factors in chronic pancreatitis. These factors seem to be much more important than thought in the past. The rare autosomal-dominant mutations N29I and R122H of PRSS1 (cationic trypsinogen) as well as the variant N34S of SPINK1 (pancreatic secretory trypsin inhibitor) are associated to a disease onset in childhood or youth. Compared to chronic alcoholic pancreatitis the progression is slow so that for a long time only signs of acute-recurrent pancreatitis are found. Only at later time points (more than 10-15 years) there is evidence for chronic pancreatitis in the majority of patients. Acute recurrent pancreatitis may therefore be regarded as a transition state until definite signs of chronic pancreatitis are detectable.

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Key words: Genetic disorders; Acute recurrent pancreatitis

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INTRODUCTION: FROM ACUTE TO ACUTE-RECURRENT AND TO CHRONIC PANCREATITIS

Pancreatitis is general is divided into acute and chronic forms. Acute pancreatitis is defined as a disease in which both exocrine and endocrine function of the gland may recover to normal (restitution ad integrum). In some patients with severe acute pancreatitis, a residual functional defect may persistent but there is no recurrent episode (Figure 1). Whereas acute pancreatitis may be related to either bile duct stones or alcohol, the main cause of chronic pancreatitis is heavy alcohol consumption[1]. Patients presenting with an acute flare of alcoholic pancreatitis often show evidence for chronic alteration of the duct, therefore already having definite signs of chronic pancreatitis. The pathogenesis of alcoholic chronic pancreatitis is under debate. Whereas one group of investigators believe that acute alcoholic pancreatitis proceeds to chronic pancreatitis, others think that this is primarily a chronic disease. It is furthermore under discussion whether alcoholic pancreatitis, when initiated, may, even at complete cessation of alcohol consumption, ever recover to normal or not.

Acute-recurrent pancreatitis may be regarded as a transition state between acute and chronic pancreatitis that is characterized by repetitive acute episodes without evidence for chronic inflammation (Figure 1). This patient group is difficult to define as we only have a description of the disease within a limited time interval. It is unclear at the moment of the investigation whether the pancreatitis resumes completely or progresses to chronic disease. Only the investigation of the long-term course of the disease will show us to what group the respective patient belongs to.

In the recent years several genetic risk factors were identified in pancreatitis[2]. In particular, mutations of the cationic trypsinogen (PRSS1), secretory pancreatic trypsin inhibitor 1 (SPINK1), and the cystic fibrosis transmembrane conductance regulator (CFTR) were described. The mutation G191R of the anionic trypsinogen (PRSS2) was shown to act as a factor protecting from pancreatitis[3]. In patients with autosomal- dominant traits the mutations N29I and R122H of PRSS1 as well as a triplication of the trypsinogen was found[4]. The other mutations were mainly found in sporadic, but also in alcohol-induced disease[5]. In this short review I will discuss the classification of hereditary or idiopathic pancreatitis, but mainly focus on the description of the course of disease in patients with genetic risk factor in order to rule out whether an acute-recurrent form exists at all. These data are mainly based on an investigation of patients and their families[6,7].

HEREDITARY OR IDIOPATHIC PANCREATITIS

The term "hereditary pancreatitis" was created in the

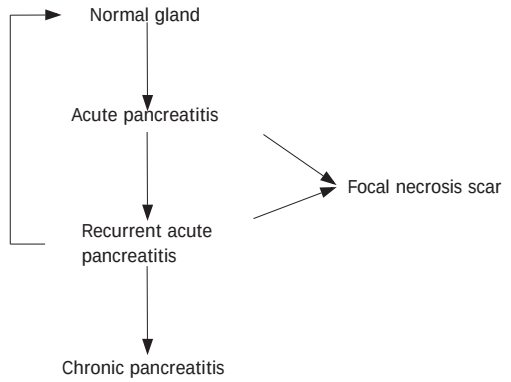


Figure 1 Relationship between acute, acute-recurrent and chronic pancreatitis.

fifties of the last century and described an accumulation of patients with pancreatitis in one family[8]. Its use is not uniform as several different definitions can be found in the literature. Most authors request 2 patients within one generation or more than 2 patients in more than one generation. Due to its origin in the pre-genetic area the term is purely phenomenological. After identification of mutations of the cationic trypsinogen (PRSS1), of the pancreatic secretory trypsin inhibitor (SPINK1) and of the cystic fibrosis transmembrane conductance regulator (CFTR) in patients with pancreatitis[2] it became evident that only less than 5% of the patients with genetic risk factors fulfil any of these definitions of "hereditary pancreatitis". In our group of patients, for example, 35% of families with autosomal-dominant trypsinogen mutations (N29I and R122H) cannot be classified as suffering from "hereditary" pancreatitis. In consequence, the definition of "hereditary pancreatitis" will have to be changed.

Another problem is the use of "idiopathic" pancreatitis in patients with genetic risk factor. Recently, even the term "familial-idiopathic pancreatitis" was created for those families having 2 members with pancreatitis[9]. Both concepts are contradictions in term as "idiopathic" means absence of any risk factor and familial accumulation or mutations always point to a risk factor. It is my suggestion to substitute the term "idiopathic" by "sporadic" that is also used in other diseases. "Autosomal-dominant" and "familial pancreatitis" (in case of absence of an autosomal -dominant trait) could be used to formally describe the familial association of the disease.

HOW FREQUENT ARE MUTATION IN PANCREATITIS?

After the first description of trypsinogen mutations several reports were published which showed up to 10% of autosomal-dominant mutations N29I or R122H in patients with "idiopathic" pancreatitis[10,11]. This is typical for an initial enthusiasm that overestimates the significance of a new finding. Later, the investigation of larger groups of patients revealed that trypsinogen mutations were present much less frequently. Interestingly, the smaller the study was, the larger were the percentages of mutations and the higher were the impact factors of the journals in which the data were published. As noted already[12] this was nice example for a publication bias favouring "positive" findings that are preferably published in journals with high impact factor.

Table 1 Mutations in different groups of patients suffering from pancreatitis

Clinical characteristic of chronic pancreatitis	% of all patients	With mutation		Without mutation
		Type of mutation	%	%
Autosomal-dominant	1	PRSS1:R122H, N29I	70-80	10-20
		PRSS1:triplication	10-20	
Familial	3	PRSS1:R122H, N29I	15	50
		PRSS1:A16V	< 5	
		SPINK1:N34S	30	
		Other SPINK1 mutations	5	
Sporadic	20	PRSS1:R122H, N29I	<< 1	15-50
		PRSS1:A16V	< 5	
		SPINK1:N34S	20-40	
		CFTR:various	20-40	
Alcoholic	75-80	SPINK1:N34S	5	85
		CFTR:various	10	
Pancreas divisum	3	CFTR:various	20	80
Tropical pancreatitis	?	SPINK1:N34S	50	50
All groups: protective mutation	1	PRSS1:G191R	In controls: 3	

Table 1 shows an overview on the frequency of the various mutations in the different groups of patients. The data largely based on our own genetic findings in more than 1000 patients with non-alcoholic chronic pancreatitis[5,12] and on several other studies[13-17]. The different groups are defined by family history (autosomal-dominant, familiar, sporadic) and the alcohol consumption. In approximately 75% of the patients with autosomal-dominant disease mutations of the cationic trypsinogen (N29I or R122H) were found. Recently a triplication of the trypsinogen was identified[4], but its frequency is unclear at the moment. A positive family history without clear autosomal-dominant trait (familial pancreatitis) predisposes to the SPINK1 mutation N34S, but also PRSS1 variants (A16V, N29I, R122H) can be seen. In patients with sporadic disease all three genes (PRSS1, SPINK1, CFTR) contribute to the risk of the disease, but, as already mentioned above, autosomal-dominant mutations are extremely rare.

In alcoholic chronic pancreatitis 15% of the patient exhibit SPINK1 or CFTR mutations[13,14]. In 22% of patients with pancreas divisium, mutations of the CFTR molecule can be identified[16]. In tropical pancreatitis half of the patients were affected from the variant N34S of SPINK1[17]. Recently Witt *et al*[3] reported the identification of a PRSS1 mutation (G191R) protecting from pancreatitis. This supports the central role of trypsin in the pathogenesis of pancreatitis. Taken together, in approximately 25% of the patients with chronic pancreatitis a genetic risk factor can be identified. A regularly updated list of PRSS1 and SPINK1 mutations can be found in the internet (http://www.uni-leipzig.de/pancreasmutation).

MUTATIONS AND RISK OF PANCREATITIS

The different mutations in pancreatitis confer a different

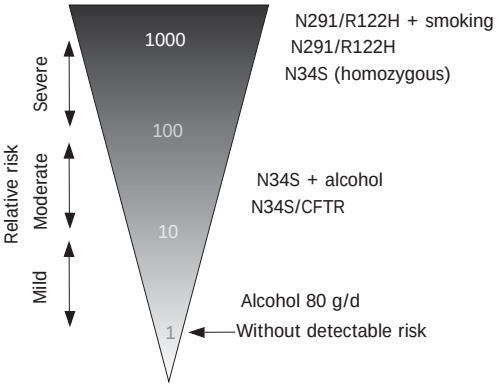


Figure 2 Strength of genetic and environmental risk factors of chronic pancreatitis.

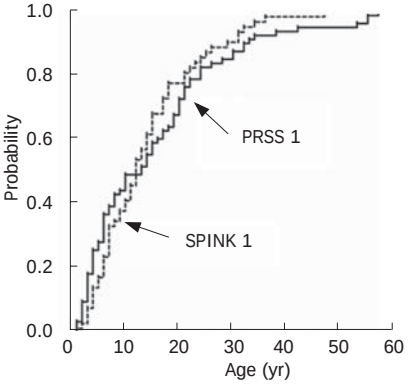


Figure 3 Age at onset of pancreatitis in patients with mutations N29I and R122H of the cationic trypsinogen and mutation N34S of SPINK1.

risk of pancreatitis. This may be calculated from the frequencies of mutations in the healthy general publication and in patients. In trypsinogen mutations N29I and R122H approximately 80% of the subjects suffer from disease[7,16]. Apart from families with autosomal-dominant disease the mutations are obviously absent in healthy subjects. At a prevalence of chronic pancreatitis of 50-100/100.000 it may be estimated that PRSS1 mutation increase the risk for pancreatitis by a factor of at least 1000-2000. The homozygous SPINK1 N34S is detected in healthy subjects at a rate of 1/10.000, and in patients with pancreatitis it is found in approximately 5%-10% of those with N34S-associated disease[19]. Therefore it may be assumed that the homozygous N34S increased the risk by a factor of about 500. The heterozygous N34S of SPINK1 as well as CFTR mutations are associated with a 20-40-fold risk of pancreatitis[18]. This is in difference to the role of alcohol that increased the risk of disease only by a factor of 3, 5-11[20]. A conclusion from these data would be that genetic risk factors confer a much higher risk than the consumption of alcohol. It may also be concluded that alcohol is a very frequent but weak risk factor of chronic pancreatitis.

Figure 2 shows an attempt for a graphical representation of these data. Autosomal-dominant trypsinogen mutations or homozygous N34S may be regarded as strong risk factors (× 100-1000). Variants of SPINK1 or CFTR confer an intermediate risk (× 10-100). Weak factors (increase up to 10 fold) like

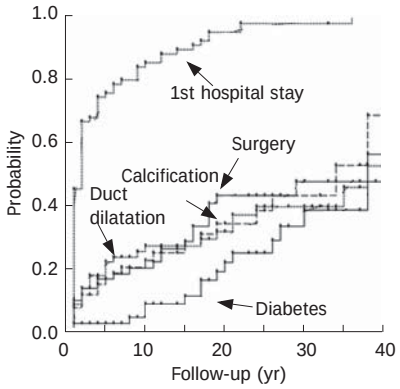


Figure 4 Progression of pancreatitis in patients with PRSS1 mutations N29I and R122H.

alcohol consumption are supplementary and may modify the course of chronic pancreatitis. The graph shows that interaction of environmental and genetic factors (i.e. N34S + alcohol or PRSS1 + smoking) further increased the probability of a disease. It is also shown that SPINK1 mutations are stronger risk factors than CFTR variants and in their homozygous form they were similar to the autosomal-dominant trypsinogen mutations. Therefore, it is no longer justified to degrade them to "disease modifier" as done recently[21].

PROGRESSION OF GENETIC PANCREATITIS

The majority of subjects with autosomal-dominant trypsinogen mutations N29I and R122H get ill in childhood[6], but this is also true for those with the variant N34S of SPINK1[7] and a considerable number of patients without any detectable risk factor ("early onset idiopathic"). The data from our patient group were shown in Figure 3[7]. The progression of disease was analysed in both patient groups. As shown in Figure 4, in the majority of the patients with the PRSS1 mutations N29I and R122H the initial event was a severe acute episode leading to treatment in a hospital. At the same time point there was no evidence for definite signs of chronic pancreatitis in the majority of these patients. Only several years later there is a slowly increasing rate of duct dilatation or calcification that may be regarded as definite prove of chronic pancreatitis. However, even after a follow-up time of 20 years after the first symptoms, only 30% of the patients show evidence for calcifications or duct dilatation. The onset of diabetes seems to be even later as after 10 years less than 10% and after 20 years less than 20% were diabetic (Figure 4). This shows that the signs of chronic pancreatitis may be absent when patients were not observed long enough.

The progression of disease in patients with N34S of SPINK1 was rather similar. There was a hospital stay early in the course of the disease, a slow appearance of duct dilatation or calcifications and a late onset of diabetes (Figure 5). Compared to patients with alcoholic chronic pancreatitis (Figure 6) the progression of disease is much slower, as in more than 50% of the alcoholics diabetes and calcification were observed already after a follow-up time of less than 15 years.

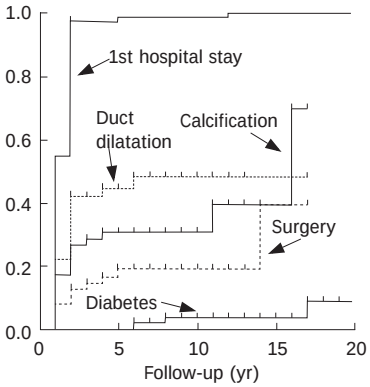


Figure 5 Progression of pancreatitis in patients with mutation N34S of SPINK1.

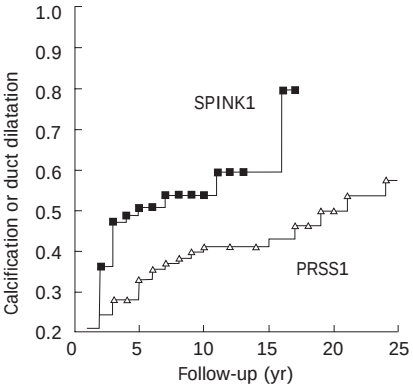


Figure 7 Probability of either calcification or duct dilatation in patients with N34S mutation of SPINK1.

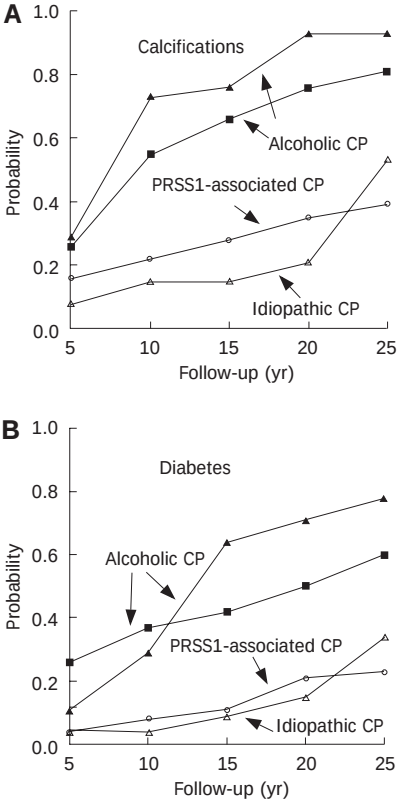


Figure 6 Progression of pancreatitis in patients with alcoholic, idopathic or PRSS1-induced disease.

DOES ACUTE RECURRENT PANCREATITIS EXIST IN PATIENTS WITH GENETIC RISK FACTORS?

The data shown above suggest that in a significant number of patients definite signs of chronic pancreatitis may be found only after a long follow-up time. From this finding it may be suspected that acute-recurrent pancreatitis is only a transition state that finally ends in chronic pancreatitis. To analyse this presence of either calcifications or duct dilatations in patients with PRSS1 (N29I and R122H) or SPINK1 (N34S) mutations were analysed by Kaplan-Meier. As shown in Figure 7 in approximately 80% of the patients with SPINK1 show at least one of the definite signs of chronic pancreatitis after a follow-up time of 15-20 years. In 50% of the patients with a PRSS1 mutation a chronic pancreatitis can be diagnosed after 25 years of follow-up. This may, however, be an underestimation of chronic pancreatitis as only two signs were evaluated. Histological specimens were available in only a few patients and the retrospective study design tends to overlook patients with chronic disease. The data suggests that recurrent acute pancreatitis is present in patients with genetic risk factors, but with time there is a slow progression to chronic pancreatitis. In the majority of patients, however, one has to wait a long time and in some others recurrent pancreatitis may persist.

CONCLUSION

The autosomal-dominant mutations N29I and R122H of PRSS1 (cationic trypsinogen), N34S of SPINK1 (pancreatic secretory trypsin inhibitor) lead to a disease onset in childhood or youth. Compared to chronic alcoholic pancreatitis the progression is low so that for a long time only signs of acute-recurrent pancreatitis are found. At later time points (more than 15-20 years) there is evidence for chronic pancreatitis (duct dilatation, calcification, histological prove of chronic pancreatitis). In patients with genetic risk factors acute recurrent pancreatitis may therefore be regarded as a transition state until definite signs of chronic pancreatitis are detectable. The progression of disease, however, is slow so that the clinical picture is compatible with an acute-recurrent pancreatitis for a very long time.

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TOPIC HIGHLIGHT

Pier Alberto Testoni, MD, Associate Professor, *Series Editor*

Endoscopic ultrasonography for evaluating patients with recurrent pancreatitis

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Abstract

Acute recurrent pancreatitis (ARP) is still a complex diagnostic and therapeutic challenge in clinical practice. In up to 30% of cases of ARP, it is not possible to establish the etiology of the disease. In the other 70%, many factors play an etiological role in ARP: microlithiasis, sphincter of Oddi dysfunction (SOD), pancreas divisum, hereditary pancreatitis, cystic fibrosis, a choledochocele, annular pancreas, an anomalous pancreatobiliary junction, pancreatic tumors or chronic pancreatitis are diagnosed. EUS should be useful in ARP as it is sensitive for diagnosing bile duct stones, gallbladder sludge, pancreatic lesions, ductal abnormalities and chronic pancreatitis. Endoscopic ultrasound (EUS) appears to be diagnostic in the majority of patients with previously unexplained pancreatitis, and offers an alternative to endoscopic retrograde cholangiopancreatography (ERCP) as the initial diagnostic test in patients with ARP.

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Key words: Acute recurrent pancreatitis; Endoscopic ultrasound

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INTRODUCTION

Acute recurrent pancreatitis (ARP) is still a complex diagnostic and therapeutic challenge in clinical practice. Defining the diagnosis itself is already problematic; it is sometimes impossible to differentiate clearly between recurrent attacks of acute pancreatitis and the early stages of chronic pancreatitis. The history and presenting symptoms may be non-specific, and as a consequence up to four out of ten patients may be initially misdiagnosed as having "non-specific dyspepsia"[1]. It is difficult to assess the significance of mild or early changes in the pancreatic duct system and the pancreatic parenchyma, particularly because the normal appearance of the pancreas already varies widely with age. In up to 30% of cases of recurrent acute pancreatitis, it is not possible to establish the etiology of the disease, despite a detailed history and extensive investigations[2], so as a result the patient is diagnosed as having "idiopathic" ARP.

In the other 70%, many factors play an etiological role in ARP; in fact, virtually any case of acute pancreatitis may lead to recurrent episodes if it is not corrected. In these patients, more extensive evaluation may lead to a diagnosis of microlithiasis, sphincter of Oddi dysfunction (SOD), or pancreas divisum. Less commonly, hereditary pancreatitis, cystic fibrosis, a choledochocele, annular pancreas, an anomalous pancreatobiliary junction, pancreatic tumors or chronic pancreatitis are diagnosed. Gullo *et al*[3] reported that 288 (27%) of the total of 1068 acute pancreatitis patients had recurrence, and alcohol was the most frequent factor.

In virtually all patients with normal upper gastrointestinal anatomy, endoscopic ultrasound EUS makes it possible to visualize the entire pancreas, the neighboring blood vessels, and the common bile duct from various scanning positions in the stomach and duodenum. This is not always possible with abdominal US because the images can be compromised by intestinal gas or fat, and the local resolution provided by the endosonographic images is unsurpassed by any other imaging method because of the close proximity of the pancreas to the stomach and proximal duodenum. In contrast to endoscopic retrograde cholangiopancreatography (ERCP), EUS makes it not only possible to view changes in the pancreatic duct, but changes in the parenchyma can also be taken into account for diagnostic considerations.

The normal endosonographic appearance of the pancreas should meet specific criteria for size, contour, parenchyma, and ducts. Wiersema *et al*[4] evaluated the endosonographic features of a small group of healthy volunteers, with no history of abdominal pain or alcohol abuse. To diagnose



Figure 1 Conventional endosonographic imaging (7.5 MHz) of choledocholithiasis. A 9.6 mm stone (A) is seen as an hyperechoic structure with acoustic shadowing (C) in the intrapancreatic tract of the common bile duct (B).



Figure 2 Common bile duct (CBD) stone with dilation of the biliary tract. PAP: Vater papilla.

pathological changes in the pancreas, it is important to take account of the normal anatomical variants. Normally, the pancreatic parenchyma is homogeneous and slightly hyperechoic. The main pancreatic duct is seen as a thin, anechoic, smooth linear structure coursing centrally through the parenchyma. There is also usually a clearly detectable difference in the echo-density of the ventral and dorsal pancreas, the ventral part being more hypoechoic[5]. Age-related changes can also vary widely. In a large prospective study, Rayan *et al*[6] found that 28% of patients with no history or symptoms of pancreaticobiliary disease had at least one pancreatic parenchymal or ductular finding similar to those seen in chronic pancreatitis. There was a tendency, albeit not significant, for the frequency of abnormalities to rise with age, particularly after the age of 60[6].

Mechanical factors promote recurrent episodes of pancreatitis by inducing a transient, or less frequently, a persistent obstruction to the flow of pancreatic juice into the duodenum, with a consequent rise in intraductal pancreatic pressure. The mechanical obstruction may also allow the bile to flow back into the main pancreatic duct with intrapancreatic activation of pancreatic zymogens; Opie first proposed this theory in 1901[7] for the pathogenesis of gallstone pancreatitis. Conditions inducing mechanical obstruction are either congenital or acquired and may be at the level of Vater's papilla, the bilio-pancreatic junction or the main pancreatic duct.

If EUS and MRCP exclude mechanical causes of pancreatitis, an autoimmune form should be considered, especially if the patient has any other "rheumatologic" conditions such as sicca syndrome, and has elevated serum IgG 4 levels. EUS shows an irregular narrowing of the pancreatic duct, with swelling of the parenchyma. There may be diffuse parenchymal enlargement, sometimes also of the pancreatic main duct. Obstructive jaundice can be a common feature. The lesion sometimes mimics a pseudotumor and differential diagnosis with adenocarcinoma becomes difficult. In autoimmune pancreatitis the Wirsung is often narrowed, but not disrupted as in cancer. Fine-needle aspiration shows lympho-plasmacytic infiltration and fibrosis, more evident in cases with a long history of recurrent pain.

CHOLEDOCHOLITHIASIS AND BILIARY MICROLITHIASIS

Pancreatitis results from gallstone disease (Figure 1) in almost 40% of cases[8]. With the US probe positioned in the genu superior, the common bile duct (CBD) can be examined in longitudinal sections as far proximally as the hepatic duct and distally to the ampulla (Figure 2). Withdrawal from the bulb under traction allows examination of the gallbladder and CBD, from its origin in the hilum to the convergence of the cystic and hepatic ducts and the proximal portion of the CBD. With this method, the hepatic duct and CBD can be visualized in 95%-100% of patients, as reported in several studies[9].

In a prospective and competitive blinded study in patients with CBD obstruction EUS gave 100% accuracy for the diagnosis of choledocholithiasis; it was also significantly more sensitive than transabdominal ultrasonography or CT[10]. In patients with unexplained ARP the use of EUS with high resolution increases the chances of detecting microlithiasis (Figure 3), a frequent cause of pancreatitis[2,11,12].

A large study by Frossard *et al* evaluated 168 patients referred with diagnosis of idiopathic pancreatitis. EUS identified an abnormality in 80% of these patients, and 62% of them had biliary tract pathology such as stones, sludge, or microlithiasis. Compared to the final diagnosis at surgery, ERCP, bile crystal analysis, or medical follow-up, EUS correctly established the causes of pancreatitis in 92% of patients[13] (Figure 4).

Microlithiasis may lead to pancreatitis through several mechanisms: transient impaction of small stones at the papilla, obstructing the pancreatic duct, or the repeated passage of stones (which may lead to papillary stenosis or SOD, both of which are associated with pancreatitis)[14]. Surgical or endoscopic treatment of occult microlithiasis significantly reduces the recurrent episodes of acute pancreatitis compared with untreated patients[5-21].

Three recent studies maintain that EUS should replace ERCP as the first procedure in patients with mild to moderate acute pancreatitis to avoid unnecessary ERCP and thus reduce ERCP-induced morbidity[22-24]. One study also suggested that the rate of morbidity and mortality

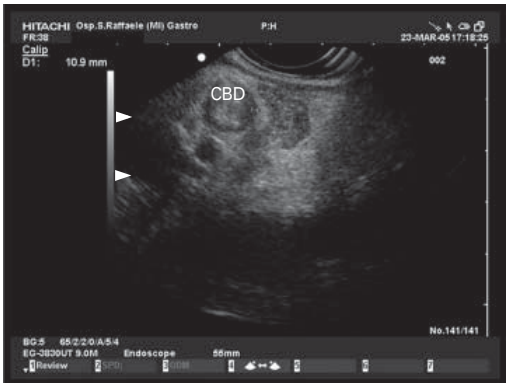


Figure 3 View of the proximal tract of the common bile duct (CBD) from the duodenum: the duct is dilated and completely obstructed by minuscule concrements (microlithiasis).



Figure 4 Hepatic ilum seen from the duodenal bulb: common bile duct with sludge (A), thickening of the CBD wall (C), and cystic duct (B).



Figure 5 Pancreas divisum. The pancreatic duct at the istmus moves towards the minor papilla (B), (A) dorsal pacreatic duct, (C) superior mesenteric vein.



Figure 6 A view of Santorini's duct (A) from the duodenal bulb, with millimetres stones in the retropapillary region (B) (minor papilla).

could be reduced by systematically using EUS in cases of acute pancreatitis, followed by ERCP with sphincterotomy when EUS has indicated CBD stones[25].

When gallstone disease is excluded as a cause of pancreatitis, EUS remains obligatory in the work-up of patients with idiopathic pancreatitis because it permits combined exploration for parenchymal and ductal abnormalities.

PANCREAS DIVISUM

Pancreas divisum (PD) is the most common congenital anatomic variant in the pancreas, present in 5%-8% of the general population, with reports of prevalence ranging from 1.3% to 7.5% in series based on ERCP findings[26-29]. PD occurs when the ventral and dorsal pancreatic ducts fail to fuse during embryogenesis. As a result, the ventral duct only drains the ventral pancreas. The majority of the pancreas then drains through the dorsal duct from the minor papilla. In these patients, the minor papilla is often stenotic and therefore slows the flow of pancreatic juice. Even relative obstruction of pancreatic exocrine secretory flow through the minor papilla can result in pancreatitis in some patients. Patients with PD and ARP respond favourably to surgical sphincteroplasty of the minor papilla or endoscopic minor papilla sphincterotomy[30].

By definition, EUS findings are indicative of PD if the course of the pancreatic duct cannot be followed from its origin at the ampulla of Vater through the ventral part to the dorsal pancreas, or if the duct cannot be visualized within the ventral pancreas. It may also sometimes be possible to visualize the separate orifices of the CBD at the major papilla and of the dorsal pancreatic duct at the minor papilla (Figure 5). Conversely, PD can be excluded if the pancreatic duct can be followed continuously from the ventral to the dorsal pancreas, or between the papilla of Vater and the pancreatic neck[31,32].

Bhutani *et al* described the possibility of accurately diagnosing PD by EUS using radial instruments[33] and suggested that divisum was present if an image simultaneously showing the portal vein, CBD, and main pancreatic duct could not be obtained[33]. In another study, Lai *et al*[32] showed that linear-array EUS appeared to be a promising, minimally invasive diagnostic imaging approach for the detection of PD, with 97% accuracy (Figure 6). Catalano *et al*, using dynamic endosonography with secretin stimulation, were able to identify patients with chronic pancreatitis and PD who subsequently benefited from endoscopic stent placement[34].

ANOMALOUS PANCREATOBILIARY JUNCTION

The CBD and PD normally unite in the wall of the



Figure 7 A malignant lesion (A) in the pancreatic head occluding the main pancreatic duct wich is dilated (B).

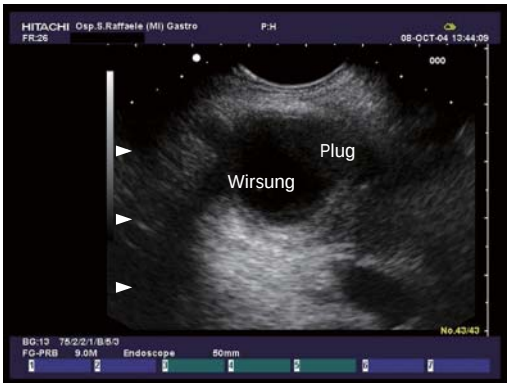


Figure 8 Dilation of the main pancreatic duct and collateral duct with evidence of plugs in the lumen in a patient with IPMT.

duodenum at the ampulla of Vater and are surrounded by a muscular sheath, the sphincter of Oddi. The ampulla is easily visualized with the probe placed in the second part of the duodenum with the balloon and the lumen filled with water, and it is seen as a clearly outlined hypoechoic structure, from which the two ducts can be followed into the pancreatic head.

An anomalous pancreatobiliary junction (PBJ) is defined by an abnormally long (> 15 mm) common pancreatobiliary channel[35]. This anomaly is associated with idiopathic ARP, choledochal cyst, and cholangiocarcinoma[36]. The sphincter does not separate the bile and pancreatic ducts, so pancreatic and bile juices may freely flow between ducts and lead to pancreatitis. ERCP and injection of the major papilla reveal the long common channel and simultaneous filling of the bile and pancreatic ducts. EUS may also establish the diagnosis.

In a prospective study Sugiyama *et al*[37] assessed the diagnostic value of EUS for anomalous PBJ. They examined 188 patients with pancreatobiliary disease, and the length of the common channel indicated by EUS was compared with that found by ERCP. EUS showed the common channel was longer than 12 mm in 88% of cases of anomalous PBJ.

ANNULAR PANCREAS

Annular pancreas is a rare congenital anomaly of the pancreas that consists of an aberrant band of pancreatic tissue arising from the head of the pancreas and either partially or completely encircling the second portion of the duodenum[38]. This abnormality is detected in 1/7000 autopsies and about 1/1500 ERCPs[39]. Adults may present with abdominal pain, ARP, or chronic pancreatitis. Although CT or MRCP may suggest the diagnosis, ERCP is usually required for confirmation. Gress *et al* described two cases where the addition of EUS aided in the diagnosis of annular pancreas visualized as "salt and pepper" parenchyma around the duodenum[40].

PANCREATIC TUMORS

Five to seven percent of patients with benign or malignant pancreatic tumors present with ARP[41]. Patients with adenocarcinoma (ADC) may suffer attacks of pancreatitis for months before cross-sectional imaging identifies a mass. EUS can be helpful in these cases but though a negative EUS result is reassuring if there is a strong clinical suspicion of ADC, it is mandatory to repeat the procedure periodically (3-6 mo) (Figure 7). Cystic lesions that cause duct compression or involvement of the main pancreatic duct tend to cause pancreatitis. EUS identified all tumoral causes in a recent series of 90 patients with undetermined causes of acute or recurrent pancreatitis[2].

Cystic neoplasms of the pancreas are traditionally organized according to the type of lining epithelium since this feature dominates the risk of malignancy and management. There are three types of mucinous lesion, benign mucinous cystadenoma, malignant mucinous cystic lesions, and intraductal papillary neoplasms. The non-mucinous lesions include serous cystadenomas and can be generally managed with observation alone. It is sometimes difficult to distinguish between serous and mucinous tumors, since the latter may contain enzyme-rich fluid, communicate with the ductal system, and may be seen more frequently in patients with symptoms of ARP. EUS can provide the means of distinguishing between serous and mucinous neoplasms by revealing a mass composed either of many tiny cysts (microcystic), with a solid-appearing hypervascular tumor, or several large cysts (macrocystic)[42].

Mucinous cystadenomas consist of one or more macrocystic spaces lined with mucus-secreting cells and are typically found in the body or tail of the pancreas. When the diagnosis of a mucinous cystadenoma is made, the lesion should be resected, on account of the high potential for malignant change.

A more frequent cause of single or recurrent episodes of acute pancreatitis is intraductal papillary mucinous tumors (Figure 8) of the pancreas (IPMT)[43]. These are villous or papillary tumors of the main pancreatic duct and/or its branches. IPMT have been subdivided (Kuroda *et al*) into a main duct and a branch duct based on the anatomical involvement of the pancreatic duct (Figure 9). EUS findings in IPMT include segmental or diffuse, moderate to marked dilatation of the main pancreatic duct, often with intraductal nodules. Pancreatic parenchymal atrophy is frequent[44] (Figure 10). Dilation



Figure 9 IPMT. A small cystic lesion (C) in retropapillary region (A), that seems to communicate with main pancreatic duct (B).



Figure 11 Normal ampulla. The ampulla (A) is seen as an hypoechoic structure from which the two ducts, the common bile duct (B) and the Wirsung (C) can be followed into the pancreatic head.

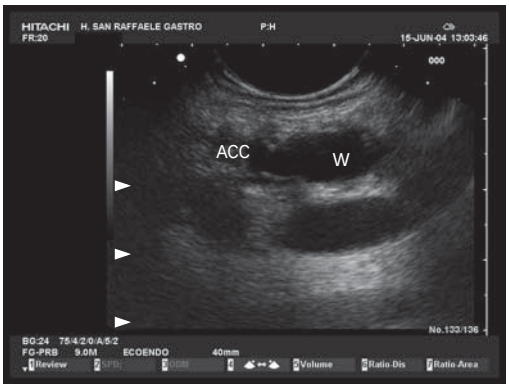


Figure 10 IPMT. Dilation of the main pancreatic duct and accessory duct. The sorrounding parenchyma is hypotrofic, hypoechoic.

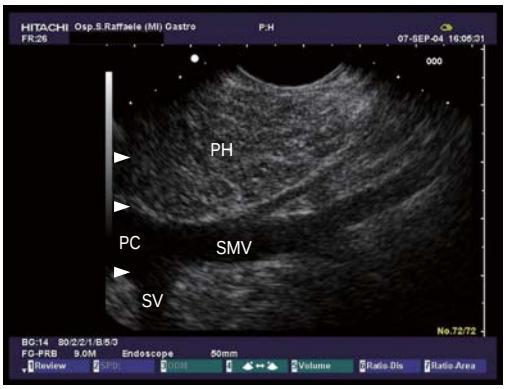


Figure 12 The pancreatic head (PH) seen from the duodenum. Minimal changes like echogenic strains in the echo texture of the pancreatic parenchyma. Portal confluence (PC) with its vessels: the superior mesenteric vein (SMV) and splenic vein (SV).

of the pancreatic duct to over 10 mm and cysts larger than 20 mm are suggestive of malignancy. EUS-FNA of the dilated pancreatic duct, including mural nodules and especially solid components of the tumor, may be helpful in the diagnosis. Histologically, the normal ductal epithelium is replaced by a mucinous metaplasia, with a broad spectrum of histopathological disorders from hyperplasia to ADC. IPMTs have been reported in elderly men, with an average age between 60 and 70 years, but can sometimes arise in younger patients. In comparison with mucinous cystadenomas, they have a lower but still relatively high malignant potential.

Ampullary tumors too-either benign or malignant - may present with ARP. Specifically, the papilla consists of the circular muscle of the CBD and an additional circular muscle enclosing the two ducts. It is seen as a hypoechoic structure from which the CBD and the main pancreatic duct can be traced and followed into the pancreatic head (Figure 11).

CHRONIC PANCREATITIS

Although patients may present clinically with ARP, many will be found, after pancreatic function tests and EUS, to have already developed some degree of chronic pancreatitis (CP). The first diagnostic endosonographic criteria of CP were suggested by Lees *et al* in 1979[45]. In 1993, Wiersema *et al*[4] refined these criteria, which can be divided into two groups: parenchymal and ductal.

Parenchymal criteria include reduced echogenicity, lack of homogeneity, loss of ventral/dorsal distinction, atrophy, hyperechoic foci, hyperechoic strands (Figure 12), and lobularity[5]. They should be sought in the dorsal gland only because the hypoechoic ventral pancreas can show features such as hyperechoic foci and strands even in normal individuals. Reduced echogenicity and lack of homogeneity are presumably caused by edema and/or reduced fat content. These changes in the dorsal gland would explain the loss of ventral/dorsal distinction. Hyperechoic foci and strands correspond respectively to small hyperechoic dots and thin, hyperechoic lines scattered in the parenchyma. When there are enough strands, they divide the parenchyma up into distinct "lobules" that may give the gland a honeycomb appearance. Information on the histologic correlates of these features is scant, but they may represent thickened fibrous deposits or may simply reflect increased visibility of normal connective tissue because of surrounding edema[46].

Ductal criteria specific to EUS include obvious to more subtle ductal dilation (> 3 mm in the head, ⩾ 2 mm in the body, > 1 mm in the tail), hyperechoic main duct margins, irregular main duct margins, and visible side branches[5].

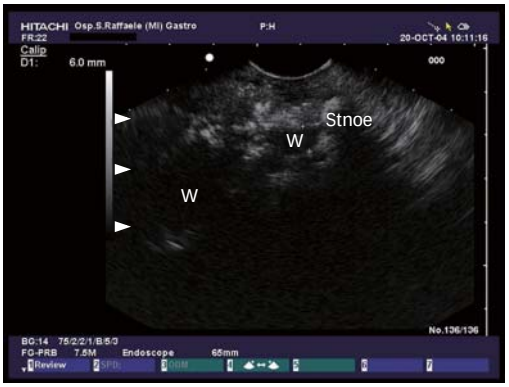


Figure 13 Advanced chronic pancreatitis with calcifications, pancreatic stone and dilation of the pancreatic duct.

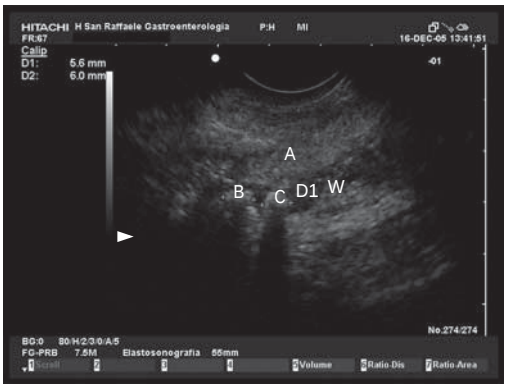


Figure 14 Pancreatic head (A) and pancreatic duct stones (B and C).

Normally, the duct walls are invisible; if they become visible, they may be considered hyperechoic.

A quantitative analysis with nine possible criteria (hyperechoic foci, hyperechoic strands, lobularity, ductal dilation, ductal irregularity, hyperechoic duct margins, visible side branches, calcifications, and cysts) suggests that, in a population at low to moderate risk of CP EUS is most reliable only when it is either clearly normal (< 2 criteria) or clearly abnormal (> 5 criteria). If three or four criteria are present, the outcome is equivocal.

With aging, the pancreas may become atrophic and appear less homogeneous, and the pancreatic duct may become slightly dilated[6]. Alcohol consumption is also associated with parenchymal changes. In a group of 19 alcohol abusers with chronic abdominal pain, 17 (89%) had chronic pancreatitis as indicated by EUS[47].

Agreement is moderate between experienced examiners in making a diagnosis of chronic pancreatitis, with either radial systems or curvilinear EUS systems. All studies agree, however, that "calcification" and "calculi" provide the highest diagnostic power (Figures 13 and 14).

If the initial workup following an attack of acute pancreatitis is negative and successive attacks occur, the evaluation has to include imaging techniques such as EUS, MRCP and blood tests, depending on individual presentation.

In conclusion, EUS should be useful in ARP as it is sensitive for diagnosing bile duct stones, gallbladder sludge, pancreatic lesions, ductal abnormalities and chronic pancreatitis. EUS appears to be diagnostic in the majority of patients with previously unexplained pancreatitis, and offers an alternative to ERCP as the initial diagnostic test in patients with ARP.

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TOPIC HIGHLIGHT

Pier Alberto Testoni, MD, Associate Professor, *Series Editor*

Sphincter of Oddi dysfunction and bile duct microlithiasis in acute idiopathic pancreatitis

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Abstract

Although there are numerous causes of acute pancreatitis, an etiology cannot always be found. Two potential etiologies, microlithiasis and sphincter of Oddi dysfunction, are discussed in this review. Gallbladder microlithiasis, missed on transcutaneous ultrasound, is reported as the cause of idiopathic acute pancreatitis in a wide frequency range of 6%-80%. The best diagnostic technique for gallbladder microlithiasis is endoscopic ultrasound although biliary crystal analysis and empiric cholecystectomy remain as reasonable options. In contrast, in patients who are post-cholecystectomy, bile duct microlithiasis does not appear to have a role in the pathogenesis of acute pancreatitis. Sphincter of Oddi dysfunction is present in 30%-65% of patients with idiopathic acute recurrent pancreatitis in whom other diagnoses have been excluded. It is unclear if this sphincter dysfunction was the original etiology of the first episode of pancreatitis although it appears to have a causative role in recurring episodes since sphincter ablation decreases the frequency of recurrent attacks. Unfortunately, this conclusion is primarily based on small retrospective case series; larger prospective studies of the outcome of pancreatic sphincterotomy for SOD-associated acute pancreatitis are sorely needed. Another problem with this diagnosis and its treatment is the concern over potential procedure related complications from endoscopic retrograde cholangiopancreatography (ERCP), manometry and pancreatic sphincterotomy. For these reasons, patients should have recurrent acute pancreatitis, not a single episode, and have a careful informed consent before assessment of the sphincter of Oddi is undertaken.

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Key words: Sphincter of Oddi dysfunction; Microlithiasis; Idiopathic pancreatitis

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INTRODUCTION

Sphincter of dysfunction (SOD) is a benign, noncalculous obstructive disorder that occurs at the level of the sphincter of Oddi (SO) (Figure 1). The pathogenesis of SOD relates either to passive obstruction at the SO caused by fibrosis and/or inflammation or to active obstruction caused by sphincter muscle spasm. These two mechanisms of functional obstruction at the SO are not mutually exclusive.

SOD is a possible cause of three clinical conditions: (1) persistent or recurrent biliary pain following cholecystectomy in the absence of structural abnormalities, (2) acute idiopathic pancreatitis, and (3) biliary pain in patients with intact gallbladders but without cholelithiasis (the least studied and most controversial clinical association). SOD also has been described in patients who have had liver transplantation, have the acquired immunodeficiency syndrome, and have hyperlipidemia. This manuscript will discuss only the association of SOD with acute idiopathic pancreatitis (AIP).

Microlithiasis is another possible cause of acute idiopathic pancreatitis. This is best studied in patients with intact gallbladders, and is felt to be one of the most common causes of AIP. Although it seems intuitive that microlithiasis may also occur *de novo* in the bile duct in patients with prior cholecystectomy, this entity is not well described.

MICROLITHIASIS AS A POTENTIAL CAUSE OF ACUTE IDIOPATHIC PANCREATITIS

Gallbladder in situ

Microlithiasis has been defined as tiny stones (1-2 mm) that are missed on imaging studies. In contrast, biliary sludge is a collection of crystals (seen only by microscopic

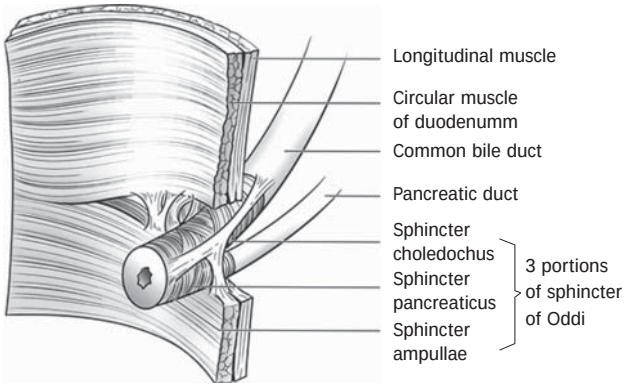


Figure 1 Sphincter of Oddi anatomy.

exam), glycoproteins, protein, cellular debris and mucin. In practice, since biliary sludge may contain microlithiasis these two terms have been used interchangeably[1]. In patients with intact gallbladders, it has been estimated that up to 60%-80% of acute idiopathic pancreatitis may be due to microlithiasis[2-4]. Other centers have found a much lower incidence of 6%-8% of "missed gallbladder disease" in AIP patients[5].

For AIP patients with an intact gallbladder and a normal transcutaneous gallbladder ultrasound, several diagnostic and therapeutic options exist. First is empiric cholecystectomy. This is still commonly practiced and is not unreasonable if the incidence of "missed stones or sludge" is common in your area. Alternatively, duodenal aspiration of bile after CCK stimulation can be utilized for diagnostic confirmation. The sensitivity of this procedure is 66%[6,7]. Direct common bile duct aspiration at ERCP has also been performed although requires CCK administration to ensure collection of gallbladder bile[8]. Technical problems with crystal analysis have limited its popularity for the confirmation of microlithiasis despite its long history of use. Endoscopic ultrasound has become a popular alternative for further diagnostic testing due to its higher sensitivity for microlithiasis and sludge[9]. (Table 1) EUS also has the advantage of evaluating several alternative causes for AIP including pancreas divisum, neoplasms, and undiagnosed chronic pancreatitis[10].

Therapy for microlithasis/sludge is generally laparoscopic cholecystectomy although endoscopic biliary sphincterotomy in the poor operative candidate[11] or long-term treatment with ursodeoxycholic acid[3] are alternatives.

Post cholecystectomy

Bile duct stones that present within 2 years of cholecystectomy are usually stones that originated in the gallbladder that were missed at the time of surgery. If they present more than 2 years after cholecystectomy, they are de novo bile duct stones. Both types can be missed on traditional imaging tests and may be small stones or "microlithiasis". Endoscopic ultrasound is the most sensitive test to detect bile duct stones and appears more cost effective and sensitive than MRCP[12]. Bile obtained from the common bile duct at ERCP for crystal analysis in patients who have had prior cholecystectomy does not have diagnostic value in AIP patients[13]. Bile crystals are rare in patients with manometrically proven sphincter of Oddi dysfunction (SOD)[14]. The fact that empiric biliary sphincterotomy benefits less AIP patients compared to dual or pancreatic sphincterotomy also suggests that biliary sludge/microlithiasis is not a likely cause of AIP in patients post cholecystectomy. Indeed the benefit of biliary sphincerotomy alone in AIP patients has been attributed to a decrease of pancreatic sphincter pressure that may occur after bilary sphincterotomy[15] rather than a treatment for microlithiasis.

Table 1 Diagnostic yield of EUS in acute idiopathic pancreatitis[10]

EUS finding	No cholecystectomy (%) (n=246)	Post-cholecystectomy (%) (n = 124)
Stones (GB or BD)	7	3
GB Sludge	11	-
Pancreas division	5	11
IPMN/neoplasm	2.8	4.8
Chronic pancreatitis (⩾ 5 criteria)	31	27
Other	4.8	4.8

SPHINCTER OF ODDI DYSFUNCTION IN AIP

Several studies have demonstrated a high frequency (30%-65%) of sphincter hypertension in patients with acute idiopathic pancreatitis, and a 50% to 87% frequency in those with chronic pancreatitis. Elevated pancreatic sphincter pressure in humans has been shown to correlate to increased intrapancreatic ductal pressure presumably playing a role in the pathogenesis of pancreatitis[16]. Whether this pancreatic duct obstruction causes the initial injury or is the result of prior pancreatic inflammation is unknown. Elevated pancreatic basal SO pressure and concomitant elevated PD pressure also occurs in patients with pain only and no prior evidence of pancreatitis arguing that it is not the sole determinant of pancreatitis. However, since pancreatic sphincter ablation does decrease future attacks of pancreatitis (although studies are primarily retrospective and uncontrolled), it appears that pancreatic SOD plays a role in the pathogenesis of recurrent attacks even if it was not the original cause.

A classification system similar to the three types of suspected biliary SOD has been proposed for possible pancreatic SOD[17]. In the contemporary proposed system, pancreatic type I patients have pancreatic-type pain, a serum amylase or lipase level of 1.1 times normal on one occasion, and pancreatic duct dilation (> 6 mm in the head and > 5 mm in the body); pancreatic type Ⅱ patients have pain and one of the preceding criteria; type Ⅲ patients have pancreatic-type pain only. This classification system is not widely utilized for several reasons: (1) Few patients with type I pancreatic SOD are reported, the vast majority of AIP patients have normal pancreatic duct caliber, (2) AIP patients would fit into the pancreatic type Ⅱ category and are the primary patients of interest, and (3) Pancreatic type pain only is not sufficiently specific to be a widely accepted diagnosis.

The gold standard for making the diagnosis of SOD

is SO manometry during ERCP. Water perfused catheters are the most commonly utilized systems although microtransducer manometry is also reported[18]. Although some experts have argued for attention to abnormalities of sphincter manometry other than baseline sphincter pressure, such as phasic wave frequency and propagation direction, only basal pressures are widely used clinically. Abnormal basal sphincter pressures are usually concordant for the pancreatic and bile ducts but may occur in only one portion of the sphincter. It has been shown that increased basal sphincter pressure is more likely to be confined to the pancreas in patients with pancreatitis and more likely confined to the bile duct in persons with elevated levels of serum liver enzymes[19].

PANCREATIC SOD TREATMENT CHOICES

Medical therapy with sphincter relaxing medications has been used in biliary SOD although data is sparse supporting their benefit. There are no trials of medical therapy in the treatment of AIP, although intravenous gabexate[20] and somatostatin[21] given during pancreatic SOM have been shown to either alter phasic wave frequency or lower pancreatic basal sphincter pressure. Endoscopic injection of botulinum toxin in patients with AIP is reported to decrease pancreatitis episodes in 80% of patients[22] although the benefit is not prolonged and there is concern about possible long-term side effects.

Endoscopic sphinctertomy results in clinical benefit for pancreatic SOD in AIP patients although the studies are mostly small case series[13,23,24]. There continues to be disagreement about the type and method of sphincterotomy. Some experts continue to perform biliary sphincterotomy alone as a first line treatment[25]. It is known that this will decrease pancreatic sphincter pressure in some patients and is thought to be a safer first line therapy. It is also a treatment for possible biliary microlithiasis. Other experts direct their endoscopic treatment to the pancreatic sphincter alone when the indication is IAP and pancreatic SOD[17]. This avoids unnecessary biliary sphincterotomy although a small one likely occurs during septotomy. Other experts advocate dual complete sphincterotomies[26]. Current literature supports temporary pancreatic stenting in all of these patients to lower their risk of procedure-induced pancreatitis. There is also some evidence suggesting a lower rate of subsequent pancreatic orifice stenosis and procedure-induced pancreatitis if the pancreatic sphincterotomy is performed with a needle knife over a pancreatic stent instead of a traction type sphincterotome[27].

Surgical sphincteroplasty has also been performed for pancreatic SOD in AIP patients[28]. Although clinical response was reported in 58% of AIP patients in this case series, it is unlikely that the more aggressive surgical approach is warranted unless endoscopic therapy is not feasible due to altered anatomy or in other unusual circumstances.

TREATMENT OUTCOMES IN IAP

There are few prospective data and only one controlled trial that address the outcome of endoscopic therapy in patients with IAP. The controlled trial used serial pancreatic stents over 9 mo *vs* serial ERCP without stent insertion and was performed in patients without sphincter hypertension so it is uncertain if the results pertain to the AIP patients with documented pancreatic SOD. The rate of recurrent pancreatitis was reduced from 53% to 11% over the 3-year study period in the stented group[29]. This positive result in manometrically normal AIP patients highlights our lack of understanding of the sensitivity and specificity of SOM.

Several case series report excellent response to biliary sphincerotomy alone[30,31] although Guelrud in an abstract found that patients who underwent dual sphincterotomy fared better (86%) than those treated by biliary sphincterotomy alone (28%)[32]. Most experts believe that treatment of the pancreatic sphincter should occur at the initial therapy although strong evidence is lacking. However, it seems rational to first incise the sphincter that correlates with the presentation of the patient, meaning that pancreatic or dual sphincterotomy should be considered the treatment of choice for AIP patients with documented pancreatic SOD. A rational approach to an AIP patient is to perform EUS to rule out other potential causes of pancreatitis and to proceed to ERCP with manometry if the patient is anxious for treatment and understands that treatment success occurs in only 60%-80% of patients and that the treatment is not without complications[33]. Some experts would limit ERCP with manometry and possible pancreatic sphincterotomy to patients with recurrent AIP since some patients may have only one attack or have attacks separated by many years.

Skeptics continue to point out that there are many problems with endoscopic therapy for AIP[34]. First, there is a variable natural history of AIP patients making it difficult to assess treatment outcome. It is clear that some patients may go years between attacks of pancreatitis making long-term follow up mandatory. Second, endoscopic therapy is not without risks even with prophylactic pancreatic duct stent placement. A prospective randomized controlled trial is needed.

CONCLUSION

Two potential causes of acute idiopathic pancreatitis are microlithiasis and sphincter of Oddi dysfunction. Microlithiasis is common in patients with an intact gallbladder and acute idiopathic pancreatitis although the reported incidence varies widely. It is best detected by endoscopic ultrasound although empiric cholecystectomy remains a reasonable choice, especially in high incidence centers. Microlithiasis has not been found to be associated with acute idiopathic pancreatitis in post-cholecystectomy patients.

Sphincter of Oddi dysfunction is also common in patients with acute recurrent idiopathic pancreatitis. It remains unclear if SOD is the initial cause of the pancreatitis or is due to prior attacks. Irregardless, sphincter ablation decreases the frequency of subsequent attacks although this conclusion is based on the few available studies that are retrospective and small. Despite

this lack of data, it is reasonable to pursue ERCP with manometry and possible pancreatic sphincterotomy in patients with frequent recurrent attacks since there are no other treatment options. However, a careful discussion with the patient of the risks versus benefits of pancreatitic sphincterotomy in acute recurrent pancreatitis is required.

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TOPIC HIGHLIGHT

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Pancreatic ductal system obstruction and acute recurrent pancreatitis

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Abstract

Acute recurrent pancreatitis is a clinical entity largely associated with pancreatic ductal obstruction. This latter includes congenital variants, of which pancreas divisum is the most frequent but also controversial, chronic pancreatitis, tumors of the pancreaticobiliary junction and sphincter of Oddi dysfunction. This review summarizes current knowledge about diagnostic work-up and therapy of these conditions.

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Key words: Pancreatitis; Congenital variants; Sphincter of Oddi dysfunction; Pancreatic tumors; Magnetic resonance cholangiopancreatography; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Acute recurrent pancreatitis (ARP) represents a clinical entity in which acute pancreatitis occurs more than once in the setting of a normal morpho-functional pancreas[1].

The diagnosis of acute pancreatitis is generally based on clinical and laboratory findings. Imaging studies are obtained to confirm clinical diagnosis, to rule out mechanical factors that may induce a transient obstruction of pancreatic juice flow, to assess the extension of the inflammatory process in and around the pancreas, and to detect possible complications.

In patients presenting with ARP and a normal gland visualized on transabdominal ultrasound and abdominal CT, a primary role for secretin-enhanced magnetic resonance cholangiopancreatography (S-MRCP) has been suggested[2].

Indeed, the exogenous administration of secretin stimulates the secretion of fluid and bicarbonate by the exocrine pancreas[3]. Consequently, the volume of fluid in the pancreatic ducts increases which permits a more accurate visualization of the pancreatic ductal system, a better detection of anatomical variants, an evaluation of the pancreatic flow dynamics and therefore an indirect assessment of the sphincter of Oddi and of the pancreatic exocrine reserve[4].

S-MRCP offers several advantages over endoscopic retrograde cholangiopancreatography (ERCP) such as its non invasiveness, the absence of procedure-related complications, the absence of contrast injection or radiation exposure and its performance in post-surgical patients. Secretin administration is safe, even in the setting of acute pancreatitis[5] but additional time and cost are required, therefore restricting the use of secretin in warranted indications i.e. assessment of ARP.

ERCP should be performed only in those patients in whom the etiology of pancreatitis cannot be achieved by S-MRCP (for inspection of the papilla, for brush cytology and biopsy sampling, for bile or pancreatic juice aspiration) or for therapeutic purposes.

An etiology can be found in 70%-80% of patients after an attack of acute pancreatitis, with alcohol abuse and gallstone disease most often implicated[6,7]. Any factor capable of causing an initial episode of acute pancreatitis has the potential to initiate recurrent episodes[2].

The purpose of this review focuses on mechanical factors that promote recurrent episodes of pancreatitis by inducing a persistent or transient obstruction to pancreatic juice flow into the duodenum with a subsequent rise in intraductal pancreatic pressure.

Potential causes of pancreatic ductal obstruction include: (1) anatomical congenital variations of the biliopancreatic ductal system; (2) acquired obstructive conditions at the level of major/minor papilla, the level of the main pancreatic duct (MPD), or the level

Table 1 Potential causes of pancreatic ductal obstruction in acute recurrent pancreatitis

Potential causes	Diagnostic evaluation patient selection	Potential treatment strategies
Congenital variants		
-PD ± Santorinicele	S-MRCP	ERCP ± EPS at the minor papilla ± transient dorsal duct stenting
-APBU	S-MRCP	ES
-Choledochocele	S-MRCP	EBS
-Type Ⅰ & Ⅳ choledochal cyst	S-MRCP	Surgical resection + HJ
-Duodenal duplication cyst	S-MRCP	Endoscopic snare resection or surgical resection
-Annular pancreas	S-MRCP	ERCP + EPS or surgical gastrojejunostomy
Acquired obstructive conditions		
-Suspected neoplasm		
ampullary tumor	EUS, S-MRCP	Curative/palliative endotherapy
MPD stricture	EUS, S-MRCP	or
IPMT	EUS, S-MRCP	surgery
-Suspected early CP	EUS, S-MRCP	ERCP + EPS ± transient MPD stenting
-Groove pancreatitis (CDDW)	EUS, S-MRCP	ERCP + dual ES + MPD stenting or surgery or somatostatin
-Pancreatic SOD type Ⅰ, type Ⅱ, type Ⅲ	S-MRCP	ERCP + dual ES + transient MPD stenting

PD: Pancreas divisum; APBU: Anomalous pancreaticobiliary union; MPD: Main pancreatic duct; IPMT: Intraductal papillary mucinous tumor; CP: Chronic pancreatitis; CDDW: Cystic dystrophy of the duodenal wall; SOD: Sphincter of Oddi dysfunction; S-MRCP: Secretin-enhanced magnetic resonance cholangiopancreatography; EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography; EPS: Endoscopic pancreatic sphincterotomy; ES: Endoscopic sphincterotomy; EBS: Endoscopic biliary sphincterotomy; HJ: Hepaticojejunostomy.

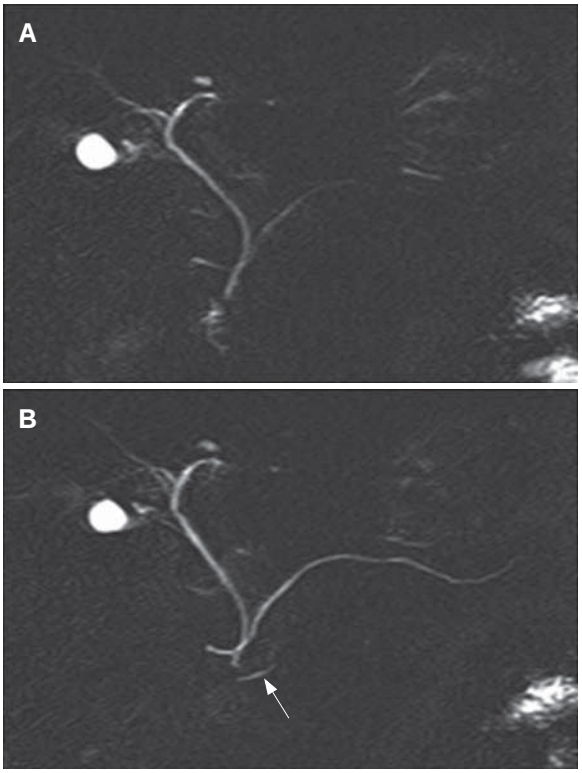


Figure 1 Normal pancreas divisum. Unenhanced (**A**) and secretin-enhanced magnetic resonance cholangiopancreatography (**B**). The ventral pancreatic duct (arrow in **B**) and the entire course of the main dorsal pancreatic duct are seen only after secretin administration.

of the duodenal wall; (3) pancreatic sphincter of Oddi dysfunction (SOD) (Table 1).

A detailed discussion of pancreatic ductal system obstruction as specific causes of ARP follows with emphasis on recent developments.

ANATOMICAL VARIATIONS OF THE BILIOPANCREATIC DUCTAL SYSTEM

Pancreas divisum

Pancreas divisum (PD) is the most common congenital variant of the pancreas, affecting 5%-14% of the Caucasian population[6,8-10]. This variant occurs when the embryological ventral and dorsal ducts fail to fuse during the second month of life in utero. In this case, the ventral duct only drains the ventral pancreas through the major papilla, whereas the majority of the pancreas drains *via* the dorsal duct through the minor papilla. The diagnosis of PD can be done safely and accurately by S-MRCP showing the dorsal pancreatic duct crossing the common bile duct anteriorly and separated from a smaller ventral duct[11] (Figure 1). A relative obstruction at the small papillary orifice of the minor papilla overburdened by draining the larger dorsal pancreas has been proposed more than 25 years ago as the mechanism of pancreatitis associated with PD[12].

However, the clinical significance of PD remains controversial. Indeed, only a minority of patients with PD (less than 5%) becomes symptomatic with ARP, chronic pancreatitis or chronic abdominal pain[10,13].

In a large ERCP series, we failed to find a significantly increased frequency of pancreatic disease among patients with PD compared with control patients[14].

Recruitment bias (greater frequency of PD diagnosis in patients referred after unsuccessful opacification of the pancreatic ductal system) may have resulted in an

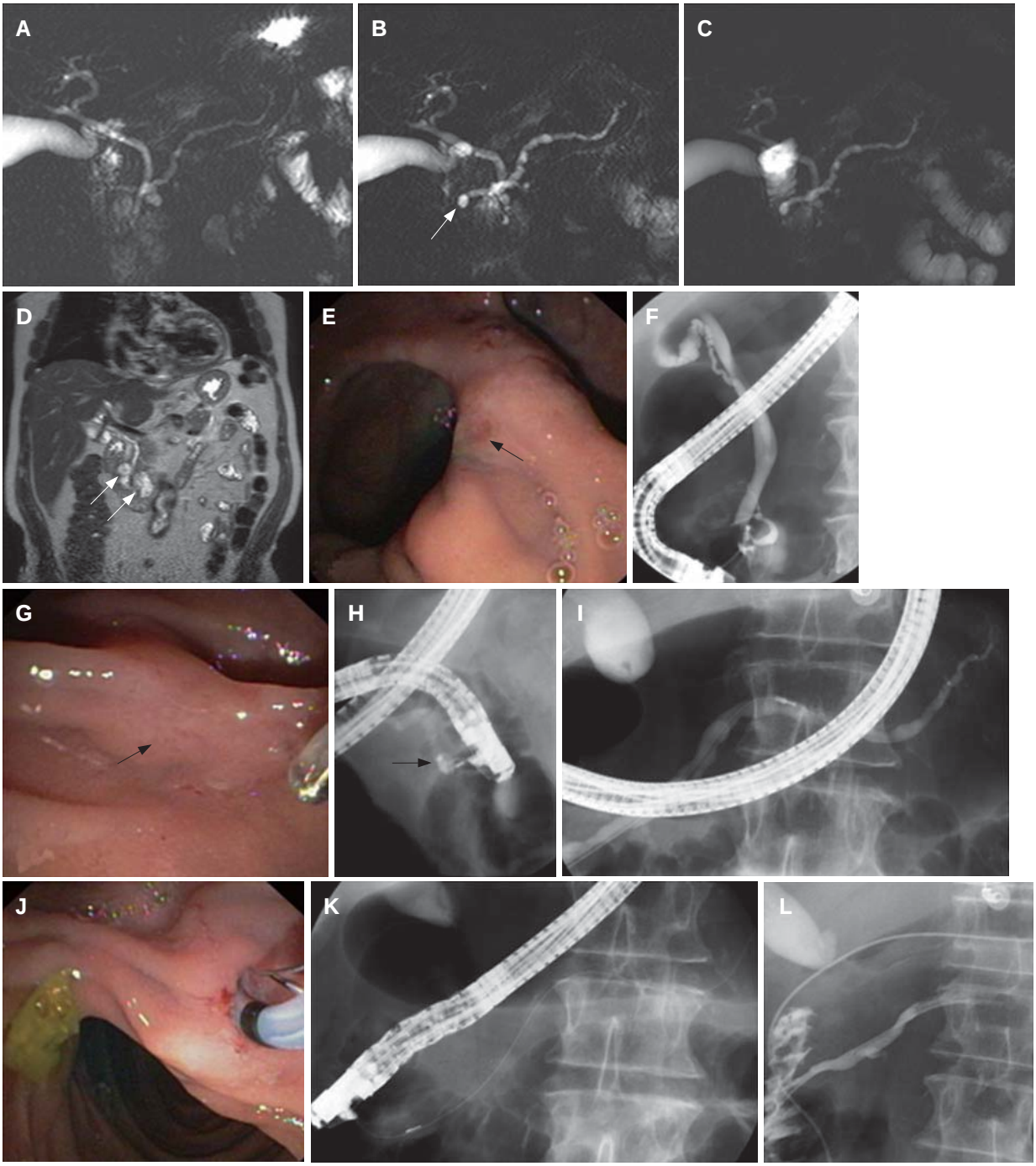


Figure 2 An 80-year-old man presented with 3 attacks of acute pancreatitis over 5 yr. There was no history of alcohol abuse, smoking, nor medications. There were no gallbladder stones at transabdominal ultrasound and no common bile duct stones at endoscopic ultrasonography. Unenhanced (**A**) and secretin-enhanced magnetic resonance cholangiography (**B**) 3 min after secretin injection and **C**: 10 min after secretin injection). Ectasia of the minor papilla (Santorinicele) (arrow in **B**) is seen only after secretin administration. Dilatation of the main pancreatic duct persists 10 min after secretin stimulation (**C**), suggesting obstruction at the level of the minor papilla. Coronal view on T2-weighted magnetic resonance imaging (**D**) shows 2 paraduodenal diverticula (arrows in **D**). The major papilla is located on a fold between 2 diverticula (arrow in **E**), whereas the minor papilla is extremely difficult to locate even after secretin injection and methylene blue staining (arrow in **G**). During ERCP, cannulation of the major papilla allows opacification of a normal common bile duct and a short ventral pancreatic duct (**F**). Cannulation of the minor papilla using the needle, in the long scope position, allows opacification of a cystic dilatation of the distal part of the dorsal pancreatic duct (Santorinicele, arrow in **H**). The main dorsal pancreatic duct is dilated (maximal diameter: 8.3 mm after correction for magnification) (**I**). Endoscopic minor papillotomy is performed with a standard pull-type sphincterotome (**J-K**) and subsequently a 6 Fr nasopancreatic catheter is inserted for 24 h (**L**). After sphincterotomy, contrast medium flows freely alongside the nasopancreatic catheter into the duodenum.

overestimation of the prevalence of PD in previous other ERCP studies investigating ARP and PD[15].

More recently, in an S-MRCP study (where the bias of referral to a tertiary center after failure of ERCP was avoided), the frequency of PD was similar in 54 control patients, in 68 patients with established chronic pancreatitis and in 157 patients in whom pancreatic disease was suspected on the basis of idiopathic acute pancreatitis (n = 67), increased serum levels of pancreatic enzymes (n = 42) or pancreatic-type pain (n = 48)[16].

As most patients with PD do not develop pancreatitis, a stenosis of the minor papilla or a Santorinicele (Figure 2)

(defined as a cystic dilatation of the distal dorsal duct just proximal to the minor papilla and considered as resulting from a combination, either acquired or congenital, of relative obstruction and weakness of the distal ductal wall) could be the additional necessary factors to cause an acute pancreatitis in a few patients with PD[17].

Identification of a Santorinicele at S-MRCP (Figure 2B) or a minor papillary stenosis (suggested by an abnormal response to secretin, i.e. persistent dilatation of the MPD greater than 3 mm at 10 min after secretin injection in patients younger than 60 years) might be helpful in selecting a subset of patients with PD and ARP who might benefit from pancreatic ductal drainage[16-18].

Indeed, patients with PD and Santorinicele showed a larger MPD in the head of the gland compared with that in patients with only PD (compare Figures 1B and 2B), both at rest and after secretin stimulation[17]. Moreover, the mean interval between the injection of secretin and the onset of duodenal filling was significantly longer in patients with Santorinicele compared with that in patients without Santorinicele[17]. These observations suggest the presence of impeded pancreatic secretion outflow at the level of the minor papilla with subsequent increased intraductal pressure.

In a prospective study including 279 patients investigated by S-MRCP, an abnormal response of the MPD to secretin stimulation could be detected in 12% of the patients with ARP but the dynamics of pancreatic duct filling after secretin stimulation did not differ between patients with or without PD[16]. Whether the observation of an abnormal response of the dorsal pancreatic duct to secretin stimulation at S-MRCP is clinically relevant still remains to be demonstrated.

There is also no consensus regarding the appropriate treatment for ARP associated with PD but available data suggest that some patients may benefit from dorsal duct drainage[19]. For example, it was reported that patients with Santorinicele may benefit from endoscopic papillotomy of the minor papilla (Figure 2G-L) as evidenced by a remarkable reduction in size of the MPD and of the Santorinicele on follow-up S-MRCP images and by symptomatic improvement[17]. The same might be true for patients with a persistent ductal dilation after secretin administration visualized at MRCP or ultrasound.

Dorsal duct drainage could be achieved by surgical sphincteroplasty of the minor papilla[20] or endoscopic therapy including minor papilla sphincterotomy, transient dorsal duct stenting or combination[13,19,21]. The overall success rate of endoscopic therapy is similar to the results of surgical sphincteroplasty. Patients with well defined bouts of pancreatitis had a significantly higher response rate (70% to 80%) to dorsal duct drainage than those with chronic pancreatitis or chronic abdominal pain[13,22].

Endoscopic minor papillotomy may be performed with a standard pull-type sphincterotome (Figure 2J-K) and subsequent placement of a 5 Fr unflanged stent into the dorsal pancreatic duct to protect against early scarring and post-ERCP pancreatitis, or with a needle knife over a previously placed dorsal pancreatic duct stent. The stent should be removed within 2-4 wk if it has not migrated spontaneously into the duodenal lumen to avoid stent-induced damage to the pancreatic duct[21].

The majority of therapeutic trials are small, retrospective case series with only one randomized controlled trial on 19 patients presenting with ARP and PD, showing a clinical benefit after dorsal duct stenting at a mean follow-up of 12 mo after retrieval of the stent[23]. However, prolonged stenting of the dorsal MPD should be avoided because of the risk of inducing pancreatic damage mainly when the ductal morphology is normal initially[22]. Complication and papillary restenosis rates were reported in 9% and 16% respectively among 69 patients with ARP and PD treated by pancreatic sphincterotomy of the minor papilla[21].

Further studies using S-MRCP should evaluate the outcome of minor papilla sphincterotomy in patients with PD and ARP in order to provide selection criteria that indicate which patients would be most amenable for therapy.

Other congenital variants

Various congenital abnormalities of the pancreaticobiliary system are associated with ARP[24]. In 1.5% to 3% of individuals there is an anomalous union of the pancreatic and bile ducts outside the duodenal wall, which results in an unusually long common channel measuring more than 15 mm proximal to the duodenum[25]. This common channel facilitates free reflux of bile and pancreatic juice into the alternative duct. Bile entering the pancreas may potentially induce acute pancreatitis by increasing intraductal pressure, and reflux of pancreatic enzymes into the bile duct predisposes to the development of the choledochal cyst[26].

Pathogenesis of ARP in cases of anomalous pancreaticobiliary union (APBU) has also been associated with temporary occlusion of pancreatic secretion by stone, protein plugs, or sphincter of Oddi dysfunction, all leading to a rise in pancreaticobiliary intraductal pressure[25]. Endoscopic ultrasonography and S-MRCP show a good overall accuracy for detecting ductal variations[27] and demonstrating associated reflux[28].

Acute pancreatitis was reported to occur in 3% to 31% of APBU patients[24]. It was usually mild and resolved in a few days with conservative treatment. Sphincter ablation by endoscopic sphincterotomy may decrease the risk of ARP[9].

Choledochocele is a rare congenital or acquired condition, depicted as a dilatation of the intraduodenal segment of the common bile duct. It was initially included in the Todani classification of the choledochal cysts as type Ⅲ[29], but recently type Ⅱ (diverticulum of the common bile duct related to a form of gallbladder duplication), type Ⅲ (choledochocele) and type Ⅴ (Caroli disease associated with congenital hepatic fibrosis) were considered as unrelated to choledochal cysts[30].

Pancreatitis develops when the cystic dilatation or its content (sludge or stones) obstruct pancreatic duct outflow. Recommended treatment consists of uprooting the choledochocele by endoscopic sphincterotomy[31].

Types Ⅰ and Ⅳ choledochal cysts (congenital dilatation of the extrahepatic bile ducts with a variable amount of intrahepatic involvement) account for more than 90% of the patients with choledochal cysts and among them, 16% presented with pancreatitis[30]. In such patients, the entire extrahepatic biliary tree and gallbladder should be removed

with reconstruction by a Roux-en-Y hepaticojejunostomy to prevent the risk of further cholangiocarcinoma.

Duodenal duplication is a cyst-like structure bulging into the duodenal lumen just distal to the papillary orifice. The duodenal duplication cysts contain Brunner's glands within their wall. The association of pancreatitis with duodenal duplication is infrequent[32]. Pancreatitis may result from occlusion of the pancreatic ductal system by the distended duodenal cyst filled with secretions or stones. Endoscopic snare resection of the top of the cyst has been described[33,34].

Annular pancreas refers to a part of pancreatic tissue partially or completely encircling the duodenum usually at the level of or just proximal to the major papilla. This anomaly occurs when the ventral bud fails to rotate with the duodenum during embryological development. This rare variant is often associated with duodenal or biliary obstructive symptoms and pancreatitis may affect the annulus or the remaining pancreas. Recommended treatment consists in gastrojejunostomy[35] in case of duodenal occlusion.

Congenital variants of the biliopancreatic ductal system provide interesting challenges when discovered during the diagnostic workup of ARP. However, most of these variants are clinically irrelevant. Therefore methods to select patients who are likely to benefit from therapy would be of the utmost importance.

ACQUIRED OBSTRUCTIVE CONDITIONS

Tumors of the pancreaticobiliary junction

It is estimated that 5% to 7% of patients with pancreaticobiliary tumors, benign or malignant, present with idiopathic ARP[9]. Mechanical blockage of the papillary orifice by ampullary tumors, may lead to painless jaundice, anemia, but also idiopathic ARP. Therefore careful papilla inspection for size and shape should be included in the diagnostic workup of ARP of unknown etiology and papilla biopsies should be taken when indicated.

Adenomas are the most common ampullary tumors and represent premalignant conditions which need endoscopic snare resection or surgical resection according to their size and histological grade[36]. Surveillance remains an option, especially for those occurring in the setting of familial adenomatous polyposis which are not symptomatic[37].

Similarly, unexplained ARP in a patient 45 years or older requires that an underlying pancreatic carcinoma be excluded[38]. The incidence of malignant pancreatic neoplasm as the underlying cause of acute pancreatitis is low (only 3%) in patients younger than 40 years of age compared to patients aged 40 to 60 years (21%) and to patients aged older than 60 years (25%)[39].

Endoscopic ultrasonography and S-MRCP are helpful procedures in assessing strictures of the MPD and associated parenchymal and ductal changes.

Cytologic and histologic intraductal sampling of pancreatic ductal strictures, tumoral molecular markers searched on pure pancreatic juice or brushing samples may help to unravel the suspicion of malignancy.

Chronic pancreatitis

ARP may also be the clinical presentation of early chronic pancreatitis (CP). Indeed, according to the recently proposed Sentinel Acute Pancreatitis Event (SAPE) hypothesis, repeated attacks of acute pancreatitis may evolve to a clinically chronic disease[40].

Early stages of chronic pancreatitis can be specifically detected by endoscopic ultrasonography (3 to 5 positive criteria among echogenic duct walls, irregular duct contour, dilated side branches, parenchymal echogenic foci or strands, lobularity of the gland,..)[41] and by S-MRCP (abnormal ductal response to secretin, progressive enhancement of the pancreatic parenchyma related to a loss of ductal or parenchymal compliance, characteristic side-branch involvement, changes in gadolinium uptake,…)[4,42].

It was shown that a substantial number of patients with ARP indeed have evidence of CP (42%-47%) during follow-up evaluation[41,43] and that CP was twice as frequent in patients with ARP compared with those with a single episode of pancreatitis[41].

Strictures of the MPD, generally due to inflammation or fibrosis around the MPD and obstructing pancreatic stones may contribute to abdominal pain or ARP in patients with CP. In these advanced stages of CP (which are not called ARP anymore), S-MRCP demonstrates the ductal anatomy, the degree and level of pancreatic duct obstruction, the associated complications (pseudocysts, common bile duct stricture) and the possible duodenal filling impairment suggesting pancreatic exocrine insufficiency. This imaging procedure is mainly useful before planning therapy and also for follow-up after relief of ductal obstruction[2].

Groove pancreatitis or cystic dystrophy of the duodenal wall is an uncommon type of CP affecting the "groove" between the head of the pancreas and the duodenum. The most characteristic finding is a sheet-like mass between the head of the pancreas and the thickened duodenal wall associated with cystic changes[44]. The cysts in the duodenal wall develop from foci of heterotrophic pancreas lying close to the pancreas. In a series of CP patients, the frequency of cystic dystrophy of the duodenal wall is to the order of 25% with a strong male predominance, a mean age of 45 years and frequent weight loss and vomiting associated to the ARP clinical presentation[45].

Endoscopic ultrasonography is the exploration of choice to search for cystic dystrophy of the duodenal wall. MR imaging can also visualize cystic formations and a band of low-intensity signals on T1 and T2 sequences, lying between the second portion of the duodenum and the pancreas, and corresponding to heterotopic pancreatic tissue.

Pancreaticoduodenectomy remains the most effective treatment since the diseased tissue is removed but endoscopic cystic and pancreatic ductal drainage and somatostatin analogs are also alternatively proposed therapies[44].

Pancreatic sphincter of Oddi dysfunction

Sphincter of Oddi dysfunction (SOD) is defined as a benign, non-calculous partial obstruction (organic or functional) of the biliary and/or the pancreatic segment of the sphincter of Oddi, giving rise to episodic upper abdominal pain or pancreatitis[46].

SOD is reported to be present in about 30% of

patients with ARP of unknown etiology[7,9]. Organic or functional obstruction of the sphincter of Oddi may promote reflux of bile into the MPD or impairment of pancreatic duct outflow, causing pancreatitis.

Patients with pancreatic SOD may be classified similarly to those with biliary SOD[6]. Pancreatic type I SOD corresponds to a papillary stenosis and is thought when pancreatic-type pain or documented ARP are associated with elevated serum amylase/lipase and a dilated MPD (> 5 mm in the body of the pancreas). In pancreatic type Ⅱ SOD, pancreatic-type pain or ARP are combined with one additional factor defining type I , and pancreatic type Ⅲ SOD includes patients having recurrent pancreatic type pain alone with none of the above findings[1,6,9].

Type I is considered to be due to a chronic inflammatory process which becomes a fibrosis with subsequent stenosis of a part or the entire sphincter. Pancreatic type Ⅱ SOD patients are thought to suffer from sphincter of Oddi dyskinesia that means a functional alteration of the physiological motility of the sphincter which causes some delay in the passage of pancreatic juice into the duodenum[47].

In suspected pancreatic type Ⅲ SOD, an objective diagnosis of dysfunction should rely on sphincter of Oddi manometry (basal pancreatic sphincter pressure > 40 mmHg) or less invasively on MRCP evaluation of changes in the size of the MPD after secretin stimulation (1). However, data regarding pancreatic SOD are sparse, making the management of this disorder even more controversial than its biliary counterpart[13].

Nowadays, sphincter of Oddi manometry tends to be replaced by the non-invasive S-MRCP[48,49] providing information regarding the sphincter of Oddi function by measuring changes in MPD diameter over time, after secretin injection. At S-MRCP, the diagnosis of pancreatic SOD may be suggested when MPD diameter remains increased by greater than 1 mm compared with baseline, throughout the 15 min testing interval. Based on MPD kinetics at S-MRCP, Mariani *et al* found concordant results between S-MRCP and sphincter of Oddi manometry in 13 of 15 patients with idiopathic ARP[48]. Moreover, a recent study suggests that S-MRCP may be useful in selecting those patients with suspected SOD who might benefit from endotherapy[49]. Interestingly, S-MRCP could replace sphincter of Oddi manometry more effectively in type Ⅱ SOD than in type Ⅲ, this latter group being the most controversial for potential therapy[49].

Available data suggest that, in suspected pancreatic SOD (on the basis of either a basal dilatation of the MPD, or an abnormal dynamic response of the pancreatic duct at S-MRCP), a dual endoscopic biliary and pancreatic sphincterotomy, whether at single or separate sessions, yields significantly better response than endoscopic biliary sphincterotomy alone[50]. However, patients with SOD have a complication rate from endoscopic sphincterotomy 5 times higher than that of patients with bile duct stones[51]. Placement of a transient pancreatic stent reduces this risk especially when the accessory duct is not patent[52]. This stent usually migrates spontaneously but in case migration does not occur, it should be removed 10-14 d following dual endoscopic sphincterotomy.

CONCLUSION

ARP should be evaluated in referral centers as diagnosis is time-consuming, usually expensive and may expose the patient to a substantial morbidity. Initial modalities of choice involve CT scan and S-MRCP to detect alterations in the pancreatic parenchyma including calcifications and tumors and in order to obtain a high resolution imaging of the pancreatic ducts.

Endoscopic ultrasonography and ERCP are the next logical steps for the detection of early CP, unappreciated malignancy and for a trial of transpapillary ductal drainage.

Future prospective trials should define which patients, with idiopathic ARP, whether or not it is associated with anatomical variants, are most likely to benefit from pancreatic ductal drainage.

These studies should allow appropriate patient selection and the development of novel, effective, preventive and therapeutic strategies to improve the clinical condition of these patients.

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TOPIC HIGHLIGHT

Pier Alberto Testoni, MD, Associate Professor, *Series Editor*

Endoscopic therapy in acute recurrent pancreatitis

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Abstract

Endoscopic retrograde cholangiopancreatography (ERCP) has evolved from a largely diagnostic to a largely therapeutic modality. Cross-sectional imaging, such as computed tomography (CT) and magnetic resonance imaging (MRI), and less invasive endoscopy, especially endoscopic ultrasound (EUS), have largely taken over from ERCP for diagnosis. However, ERCP remains the "first line" therapeutic tool in the management of mechanical causes of acute recurrent pancreatitis, including bile duct stones (choledocholithiasis), ampullary masses (benign and malignant), congenital variants of biliary and pancreatic anatomy (e.g. pancreas divisum, choledochoceles), sphincter of Oddi dysfunction (SOD), pancreatic stones and strictures, and parasitic disorders involving the biliary tree and/or pancreatic duct (e.g Ascariasis, Clonorchiasis).

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Key words: Acute pancreatitis; Endoscopic retrograde cholangiopancreatography; Endoscopic Ultrasound; Choledocholithiasis; Ampullary lesions; Cystic tumors of the pancreas; Sphincter of Oddi dysfunction; Pancreas divisum; Choledochal cyst; Pancreatic stones

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INTRODUCTION

As endoscopic retrograde cholangiopancreatography (ERCP) has evolved from a mainly diagnostic to a predominantly therapeutic modality, so have the indications for pancreatic endotherapy. ERCP is no longer a "first line" investigation but used selectively for patients identified by less- or non-invasive imaging techniques to have a problem that may benefit from endoscopic therapy. Idiopathic acute recurrent pancreatitis (ARP) is a frequent reason for referrals to ERCP endoscopists[1,2]. Conditions causing ARP amenable to endoscopic diagnosis and treatment include: (1) Choledocholithiasis, including microlithiasis ("biliary sludge"); (2) Congenital abnormalities of biliary and pancreatic anatomy (e.g. choledochocele, duodenal duplication/diverticulum, pancreas divisum); (3) Ampullary lesions, benign and malignant; (4) Mucin-secretin pancreatic tumors, including cystic neoplasms in communication with the pancreatic duct, and intrapancreatic mucin-secretin neoplasms (IPMNs); (5) Pancreatic duct strictures, stones and neoplasms; (6) Sphincter of Oddi dysfunction (biliary and pancreatic); (7) Biliary and pancreatic parasites.

CHOLEDOCHOLITHIASIS, INCLUDING BILIARY SLUDGE (MICROLITHIASIS)

In the West, bile duct stones (choledocholithiasis), including microlithiasis (biliary sludge), are by far the commonest cause of ARP. Cholecystectomy will usually take care of the problem. However, when ARP patients have apparently normal gallbladders without stones on cross-sectional imaging, the diagnosis is often questioned. Transabdominal ultrasound (TUS) provides useful information about gallbladder wall and the diameter of bile ducts, but lacks sensitivity for small stones (< 5 mm) and biliary sludge. Endoscopic ultrasound (EUS), a less invasive test than ERCP, is an excellent tool for diagnosing microlithiasis[3]. In my practice, we frequently schedule EUS with ERCP available immediately afterwards (under the same sedation) when we are looking for bile duct stones in patients with recent acute pancreatitis or biliary colic with transiently elevated liver tests, or assessing obstructive jaundice. If the EUS is negative for stones or ampullary lesions, we do not proceed to ERCP. Typically, small gallstones migrate spontaneously out into the small intestine within 24 h. If the liver tests (e.g. bilirubin, alkaline phosphatase, alanine and aspartate aminotransferase (ALT, AST) normalize quickly, and the bile duct resumes a normal diameter, it is very likely that the offending stone has passed. Patients with an intact gallbladder who can be shown by EUS to have microlithiasis should be offered cholecystectomy. Not all surgeons believe that microlithiasis is a satisfactory

explanation for gallstone-like symptoms, but the results of cholecystectomy are convincing to this author! Is there a role for "empiric" ("best guess") biliary sphincterotomy in patients with ARP who have an intact gallbladder but cannot be shown by EUS to have stones or biliary sludge? This intervention would also address biliary sphincter of Oddi dysfunction (SOD) (see below). Preserving the sphincter of Oddi is important in young patients. However, elderly patients with comorbidities that render them "high risk" for surgery may be safely managed by empiric sphincterotomy for symptomatic choledocholithiasis, whether or not the offending sludge or stones can be identified by other imaging[4]. Oral dissolution therapy (i.e. ursodeoxycholic acid) for suspected biliary sludge seems a logical alternative to endotherapy in ARP. However, as the sludge is rarely comprised of pure cholesterol the results are unpredictable and often disappointing.

CONGENITAL ABNORMALITIES OF BILIARY AND PANCREATIC ANATOMY

Choledochal cysts are congenital abnormalities of the biliary tree[5]. Five types are recognized by the Todani classification. Fusiform dilatation of the extrahepatic bile duct (Type Ⅰ) is the commonest choledochal cyst encountered by ERCP endoscopists. Choledochoceles (Type Ⅲ) involve the distal bile duct and protrude as a symmetrical, smooth-surfaced mass into the duodenum at the expected level of the main duodenal papilla. Choledochoceles often are associated with anomalous pancreatico-biliary ductal drainage, such that the patients are predisposed to develop ARP. Blind-ending choledochoceles drain poorly, accumulating sludge and stones, which cause a variety of problems, including cholangitis, obstructive jaundice and gallstone pancreatitis. All choledochal cysts carry some risk of malignant transformation (to cholangiocarcinoma), but this is considered minor for choledochoceles (Type Ⅲ cysts), which are effectively treated by exposing the cyst lumen (usually by needle knife papillotomy) and thereby improving bile flow. Another congenital defect that can interfere with bile and pancreatic juice flow is a duplication cyst of the descending duodenum. This is amenable to endoscopic therapy: a needle knife is used to incise the typical septum that is present. A unique form of duodenal diverticulum - the so-called "windsock" diverticulum, is also a risk factor for ARP. This "billowing" duodenal diverticulum can be managed endoscopically. The commonest abnormality of pancreatic duct anatomy seen in routine ERCP practice is pancreas divisum (P Div), in which the dominant drainage of the pancreas (the dorsal duct) is through the minor duodenal papilla[6]. The ventral pancreatic duct (PD) in P Div is typically small and may be "invisible" (the unsuspected presence of a tiny ventral duct is a common cause of a "failed pancreatogram". The P Div anomaly occurs in 7%-8% of American of European ("Causasian") descent. It is much rarer in African Americans, Asians, Hispanics and other non-Caucasian ethnic groups. P Div is certainly associated with ARP but why a few patients are affected while the majority are spared is unknown. Several studies have suggested that a heterozygous defect in the cystic fibrosis transmembrane regulator (CFTR) gene may predispose patients with P Div to ARP[7]. Improving the drainage of the dorsal PD by minor papillotomy stops the attacks in 70%-80% of cases if chronic pancreatitis (CP) is not already established. Once CP is present, minor papillotomy may only benefit 40%-50%. Patients with a "pancreatic pain syndrome" and P Div but no other objective evidence of pancreatitis do even worse, with symptomatic benefit in only 20% or so. Annular pancreas is a congenital defect resulting in an incomplete ring of pancreas encircling the descending duodenum. This is said to be a cause of ARP, but as annular pancreas is frequently associated with P Div, the predisposition to pancreatitis is more likely a manifestation of the latter. Patients with annular pancreas may exhibit partial gastric outlet obstruction (from constriction of the descending duodenum by a swollen pancreas) during attacks of pancreatitis.

AMPULLARY LESIONS, BENIGN AND MALIGNANT

Ampullary adenomas and carcinomas create an obstruction to pancreatic exocrine secretion and bile flow[8]. Duodenoscopy is mandatory in the investigation of ARP. This author believes that every trainee in gastroenterology should learn to use a side-viewing duodenoscope, not for ERCP but to be able to assess duodenal lesions, including those involving the major and minor papilla. For adenomas and cancers limited to the mucosa (T1 lesions) by endoscopic ultrasound (EUS) examination, endoscopic resection is an option[9]. The main duodenal papilla is a site of predilection for polyp formation in both sporadic and familial adenomatous polyposis (FAP). Polyps < 3 cm in diameter can be removed endoscopically without prior EUS. This is usually accomplished by hot snare cautery, followed by stenting of the pancreatic duct orifice to reduce the risk of procedure-related acute pancreatitis[10]. Piecemeal resection is also an option, but carries a higher risk of recurrence. Polyps ⩾ 3 cm should be assessed by EUS looking for extension of adenomatous tissue into the bile duct and/or pancreatic duct, and to determine the depth of invasion should tumor be present. Clearly malignant tumors of the duodenal papilla (ampullary cancers) are not suitable for endoscopic resection. Biliary and pancreatic drainage may be improved by papillotomy. However, biliary stenting without papillotomy is a safer way to treat obstructive jaundice in ampullary cancer, as these lesions are often quite vascular and bleed when incised. Polyps and tumors involving the minor duodenal papilla rarely cause ARP. However, a cystic dilatation of the distal (duodenal) end of dorsal pancreatic duct in pancreas divisum, called a Santorinicele, may indicate obstruction to flow in patients with P Div and ARP[11]. Incision of the Santorinicele with a re-establishes good flow and may resolve the clinical problem.

CYSTIC TUMORS OF THE PANCREAS, INCLUDING MUCIN-SECRETING CYSTADENOMAS AND IPMNS

Cystic tumors of the pancreas may present with ARP[12]. Serous cystic (or microcystic) tumors have a typical "honeycomb" appearance due to clusters of tiny

cysts. Microcystic adenomas rarely undergo malignant transformation. On the other hand, mucinous cystic tumors can undergo malignant transformation. These tumors may be difficult to distinguish from pancreatic pseudocysts on cross-sectional imaging, but EUS with fine needle aspiration (FNA) of the contents usually makes the diagnosis. About 50% of mucinous cysts of the pancreas have high levels of carcinoembryonic antigen (CEA), the fluid stains positive for the presence of mucin, viscosity is increased (> water) and amylase levels are generally normal or much lower than would be expected in a communicating pseudocyst. The cyst aspirate is also sent for cytologic examination to look for signs of malignant transformation (to cystadenocarcinoma). It used to be said that cystic tumors never communicated with the pancreatic duct (PD), but this is patently untrue. Mucinous cysts communicating with the duct may cause it to become quite dilated. Another source of mucinous distension of the PD is the presence of small tumors of the PD side branches (intrapancreatic mucin-secretin neoplasm, or IPMN)[13,14]. Typically, these present with ARP and imaging showing gross dilatation of the PD. EUS may also reveal nodules within the main PD or side branches. Pancreatoscopy using "mini-'scopes" may identify villous-looking tumors within the PD. At endoscopy with a side-viewing instrument, the main papilla may appear to "wink" open and discharge gelatinous material. Due to the malignant potential of mucinous tumors of the pancreas, surgery is the treatment of choice. In patients unfit for surgery, periodic aspiration of large cysts may reduce the frequency of ARP. Endoscopic stenting of PD is rarely helpful in IPMN as the stent lumen quickly becomes occluded with mucus. Other cystic tumors of the pancreas may occasionally present with ARP (e.g. neuroendocrine tumors which have undergone cystic degeneration, cystic metastases).

PANCREATIC DUCT STRICTURES, STONES AND NEOPLASMS

Pancreatic endotherapy is becoming important in the management of a number of mainly benign pancreatic disorders[15-17]. Strictures of the main PD may be benign or malignant. Benign strictures are usually seen in chronic pancreatitis, but may form after trauma (blunt or penetrating) or instrumentation (e.g. duct stenting). Benign strictures may be solitary or multiple, the latter creating the so-called "chain of lakes" or "string of pearls" appearance. Pancreatic stones frequently form in association with PD strictures, presumably reflecting poor drainage of exocrine secretions. Often these stones are an epiphenomenon, and not truly obstructing. Should pancreatic duct stones be removed? Certainly, it is tempting to try to clear the duct of stones in symptomatic patients, but the results are often disappointing. Pain in chronic pancreatitis is often multifactorial. Endotherapy of presumed obstructing stones and benign PD strictures may be considered a "trial of therapy": if the patient gets symptomatic relief, formal surgical drainage of the pancreas (e.g. by lateral pancreaticojejunostomy, or Puestow procedure) with or without parenchymal resection may help the patient long term. After pancreatic sphincterotomy, small pancreatic stones can usually be removed using a basket or balloon catheter. Large stones that completely obstruct the lumen cannot be recovered this way, and require contact or extracorporeal shockwave lithotripsy (ESWL) to fragment them first[18]. The Brussels group (Drs Deviere and Cremer) have reported excellent results using ESWL for PD stones. PD strictures are typically managed by endoscopic stenting. There are few data on metal mesh stenting in benign pancreatic disease, although several European studies have suggested benefit. At present, metal mesh stenting is not recommended in the US for the management of benign PD strictures[19]. Plastic pancreatic duct stents can induce focal pancreatitis and strictures by occluding small side branches. The recently-introduced "star-shaped" stents that facilitate flow of pancreatic secretions around them may address this problem. As migration of plastic stents into the PD creates a difficult management problem, non-flanged stents intended to spontaneously dislodge into the bowel are made with a curl - or "pigtail" - at their distal end. An interesting application of metal mesh stents in benign pancreatic disease has been tried by the ERCP group from Indiana University (Drs Lehman and Sherman), with some success. They had a group of P Div patients with ARP who failed to improve despite repeated minor papillotomies. As their dorsal PDs were not dilated, they were considered unsuitable for surgical drainage procedures. Drs Lehman and Sherman used expandable metal mesh stents inserted through the minor papilla to cause the dorsal PDs to progressively dilate over time[20]. After this treatment, surgeons were able to perform lateral pancreaticojejunostomies on these patients. Pancreatic cancer (adenocarcinoma) is an unusual cause of ARP, but it should always be considered in elderly patients with unexplained bouts of pancreatitis. EUS may identify small lesions in the PD before a mass is appreciated on CT or MRI scan. Pancreatic stents are rarely indicated in pancreatic cancer, but there have been reports of patients with intractable pain or steatorrhea benefiting from relief of malignant PD obstruction. As mentioned previously, cystic neoplasms, IPMNs and neuroendocrine tumors of the pancreas that can present with ARP.

SPHINCTER OF ODDI DYSFUNCTION (SOD)

When no other cause of ARP is identified in a patient with prior cholecystectomy, SOD should be considered[21,22]. At present, the only way to measure biliary and pancreatic sphincter pressures is perform ERCP using a manometry catheter[23]. Historically, this has been associated with increased risk of post-ERCP pancreatitis (compared to non-manometry ERCP). As Freeman has pointed out, it is not the manometry itself that is dangerous but the instrumentation of a hypersensitive sphincter and (typically) non-dilated bile duct and pancreatic ducts[24]. Prophylactic stenting of the PD after manometry in cases of suspected SOD has significantly reduced the risk of post-ERCP pancreatitis (PEP), and virtually abolished severe (necrotizing) PEP. Most experts now recommend same-session biliary and pancreatic sphincter pressure measurements in ARP patients, as 60%-70% of those

with elevated biliary sphincter pressure have concurrent pancreatic sphincter hypertension[25]. That is, if only biliary pressures are measured and sphincterotomy performed for sustained elevations in ARP, more than 1/2 of these patients will continue to have attacks of pancreatitis. The exact mechanism of ARP in sphincter of Oddi dysfunction (SOD) is unknown[26]. Pharmacologic maneuvers aimed at reducing sphincter of Oddi (SO) pressure, including nitrates and calcium channel blockers, have been disappointing. One small study using intrasphincteric botulinum toxin injection claimed an 80% success rate (12 of 15 patients) for relief of ARP[27], but others have failed to reproduce this impressive outcome. Stenting the PD orifice alone (i.e. without sphincterotomy) is not helpful in SOD: it is likely to exacerbate rather than improve pancreatic pain in the setting of a hypertensive sphincter.

BILIARY AND PANCREATIC PARASITES

A rare cause of ARP, usually limited to tropical climates, is the presence of parasites within the bile ducts or pancreatic duct. Certain liver flukes (e.g. Fasciola hepatica[28], Clonorchis sinensis[29]) like to live and lay their eggs in the bile ducts, where they cause irritation and obstruction (especially when they die). Ascaris lumbricoides worms like to live in the pancreatic duct, where they obstruct flow of exocrine secretions[30]. In the North of India (Kashmir), Ascariasis is the commonest cause of acute pancreatitis. The treatment of these parasites is principally pharmacologic, but occasionally the ERCP endoscopist is called upon to decompress ducts obstructed by parasites, usually by sphincterotomy and trawling (by balloon or basket catheter).

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TOPIC HIGHLIGHT

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Diagnosis and management of relapsing pancreatitis associated with cystic neoplasms of the pancreas

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Abstract

One of the most important causes of relapsing pancreatitis is a cystic neoplasm of the pancreas. These low grade malignancies may cause pancreatitis by obstructing or communicating with a pancreatic duct. Patients with relapsing pancreatitis and a focal fluid fluid collection should be investigated for the possibility of a mucinous cystic neoplasm. Cross sectional imaging can provide a diagnosis with the imaging findings of a low attenuation cystic lesion containing mural calcification (CT scanning) or a lobular T2 enhancing lesion (MRCP). Endoscopic ultrasound can provide more detailed imaging with the ability to guide fine needle aspiration of the cyst fluid. Cyst fluid analysis can provide a diagnosis of a mucinous cystic lesion with the combination of cytology (mucinous epithelium), elevated carcinoembryonic antigen (CEA), and the presence of DNA mutations. Management of these patients consists of surgical resection and monitoring in patients not able to withstand surgery.

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Key words: Pancreatitis; Relapsing pancreatitis; Endoscopic ultrasound; Cystic neoplasms; Intraductal papillary mucinous neoplasms; Fine needle aspiration

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INTRODUCTION

Acute and chronic relapsing pancreatitis may be caused by a number of structural abnormalities of the pancreas. Of the malignant and pre-malignant lesions of the pancreas associated with pancreatitis, cystic and intraductal tumors are the most common. Patients with these lesions may experience pancreatitis at the time of diagnosis or suffer from relapsing pancreatitis throughout their medical illness. Approximately 9% of patients with recurrent pancreatitis will have an associated pancreatic malignancy[1]. The most likely cause of malignancy-associated pancreatitis is ductal obstruction. Since intraductal papillary mucinous tumors commonly involve the main pancreatic duct and its side branches, recurrent pancreatitis is particularly common in these patients[2].

CLINICAL PRESENTATION

Patients with relapsing pancreatitis present with discrete episodes of abdominal pain often accompanied by nausea and vomiting[3]. After the initial clinical evaluation with laboratory testing, cross sectional imaging is obtained in order to confirm the diagnosis and to determine the extent and severity of the pancreatic inflammation.

The most common abnormality encountered on cross sectional imaging in patients with relapsing pancreatitis is a cystic lesion or a focal fluid collection[4]. In this context, the chief differential is a pseudocyst versus a cystadenoma of the pancreas[5]. Pseudocysts represent a focal collection of inflammatory fluid, leaking from the pancreas. Pseudocysts may persist after resolution of pancreatic necrosis, and the presence of a ductal stricture may contribute to persistence[6]. In contrast, a cystadenoma of the pancreas represents a benign, pre-malignant, or malignant cyst and may also be responsible for the episode of pancreatitis[7]. It is often difficult to determine if a focal fluid lesion is responsible for the episode of pancreatitis or is a consequence of the pancreatitis (pseudocyst). In general, focal fluid collections that are surrounded by a well-defined, thin-walled rim of tissue are more likely to be a form of malignancy, in contrast to thick-walled pseudocysts containing inflammatory debris.

Clinically, there are few clues to differentiate between focal inflammatory fluid collections and cystic neoplasms. However, the findings of jaundice and the recent onset of diabetes should raise the possibility of occult malignancy[8]. Pseudocysts may become apparent with complications

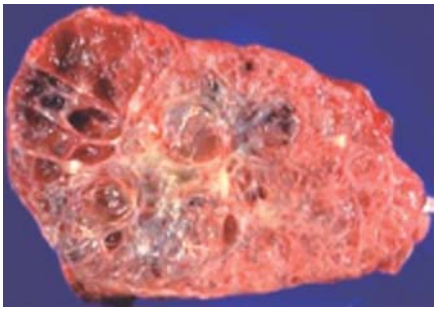


Figure 1 Gross surgical pathology specimen of a serous cystadenoma. Note the presence of a honeycomb appearance of the lesion.

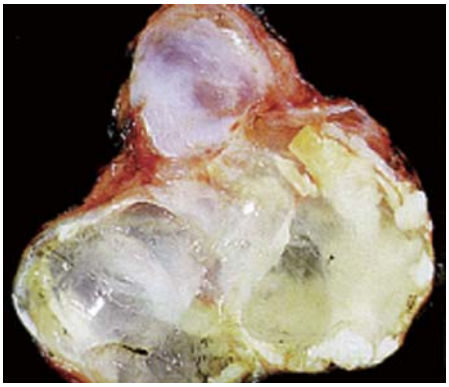


Figure 2 Gross surgical pathology specimen of a mucinous cystadenoma. The cyst cavities are filled with a clear viscous fluid.

such as hemorrhage or infection. In general, small cystic lesions are rarely responsible for chronic abdominal symptoms.

PATHOGENESIS

The pathogenesis of cystic neoplasms of the pancreas is based on mutations in the epithelial cells lining the pancreatic duct[9]. Serous cystadenomas are strongly associated with mutations of the Von Hippel Lindau (VHL) gene. The VHL gene is likely to play an important role in the pathogenesis of sporadic serous cystadenomas. In one study, 70% of the sporadic serous cystadenomas studied demonstrated a mutation in the VHL gene which probably results in hamartomatous proliferation of these centroacinar cells[10].

The pathogenesis of mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN) involves a separate set of mutations. K-ras mutations are present only in mucinous cystic neoplasms and not in serous microcystic adenomas. The frequency of K-ras mutation in mucinous cystic neoplasms is linearly related to the grades of atypia and the imposition of other mutations is thought be important in the pathogenesis of malignancy. The detection of mutations in tumor suppressor genes is the basis of a newly developed cyst fluid diagnostic test[11].

PATHOLOGY

Serous cystadenomas are benign, solitary, cystic tumors that arise from centro-acinar cells (Figure 1). Microcystic serous cystadenomas are composed of multiple small thin-walled cysts with a honeycomb-like appearance on cross section[12]. The most characteristic finding is a central fibrotic or calcified scar. Macrocystic serous cystadenomas are composed of far fewer cysts, and the diameter of each cyst varies from microcystic to large cavities[13]. The presence of discrete, large cystic cavities mimics the appearance of mucinous lesions. However, the cyst fluid from serous cystadenomas is non-viscous, contains low concentrations of tumor markers and may contain blood as a result of the vascular nature of the lesions[14].

Mucinous cystic neoplasms (MCNs) are composed of discrete individual cystic compartments that vary in diameter. The epithelium lining MCNs is a mucinous columnar epithelium (Figure 2). The World Health Organization classification catalogues MCNs into three types, based on the degree of epithelial dysplasia: benign, borderline, and malignant[15]. The degree of atypia of the tumor is classified according to the most advanced degree of dysplasia/carcinoma present.

Beneath the lining of MCNs lies a band of ovarian tissue. Many authorities have restricted the very definition of MCNs to include only those cystic mucinous tumors that contain ovarian stroma[2]. The role of the ovarian tissue in the pathogenesis is not known. However, when this definition is used, MCN's are rarely found in males.

IPMNs are similar to MCNs in that they are cystic tumors that secrete mucin into the cystic cavities. However, IPMNs are characterized by a unique papillary epithelium and arise from the ductal epithelium. The presence of a papillary neoplasm infiltrating the ductal lining causes the pancreatic duct to dilate. The presence of the tumor itself is a cause of duct dilation, as well as the obstruction induced by the papillary fronds and mucus globules. Mucin production may be so excessive that mucin may be spontaneously extruded through the pancreatic sphincter and out of the ampulla. The degree of dysplasia exhibited by the epithelium may range from mild to moderate to severe (carcinoma *in situ*), and the foci of early malignancy may be evident by the presence of mural nodules[16]. The solid malignancies that arise from IPMN are more likely to have papillary features, as compared to typical pancreatic malignancies that arise from the main pancreatic duct.

CLINICAL PRESENTATIONS

Most patients with a pancreatic cystic lesion have non-specific symptoms[22]. The cystic lesion is usually found with CT or US imaging performed for the evaluation of another condition. When symptoms are present, the most common presentation is recurrent abdominal pain, nausea, and vomiting as result of mild pancreatitis[17]. Cystic lesions that cause duct compression or involvement of the main pancreatic duct are prone to cause pancreatitis. Chronic abdominal pain and jaundice are a rare presentation of a cystic lesion and suggests a malignancy or a pseudocyst. Patients with a cystic malignancy will present with symptoms and signs similar to pancreatic cancer, i.e. pain, weight loss, and jaundice[18]. Pseudocysts may arise after an

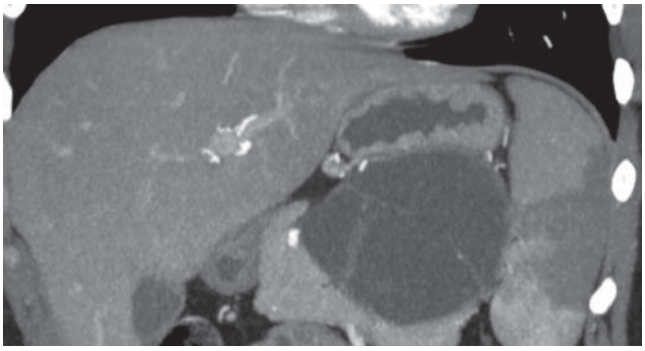


Figure 3 Reformatted CT demonstrating a mucinous cystic neoplasm indenting the stomach. Note the presence of septations.

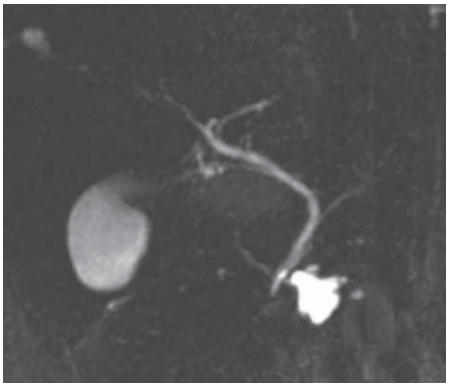


Figure 4 MRCP of side branch IPMN.

episode of acute pancreatitis or insidiously in the setting of chronic pancreatitis and are associated with chronic abdominal pain. It is common for cystic lesions associated with pancreatitis to be diagnosed as pseudocysts and be confused with cystic neoplasms that also cause pancreatitis.

DIFFERENTIAL DIAGNOSIS

The differential diagnosis of a cystic lesion of pancreas is very wide and often causes confusion. Since the treatment of a pseudocyst and cystic neoplasm are so different, it is incumbent on the clinician to first differentiate between these major categories of lesions. Although it is unusual for a patient with a pseudocyst to present without preceding symptoms, it may occur in mild chronic pancreatitis. Evidence of inflammatory changes or calcifications in the pancreas is suggestive of a pancreatic pseudocyst. However, in the initial setting of mild pancreatitis it may be difficult to differentiate between a cystic neoplasm that has caused pancreatitis and a small pseudocyst that has formed as a result of pancreatitis. If a cystic lesion has been present for many years, it is highly likely that the lesion represents a cystic neoplasm. Congenital cysts of the pancreas are rare[19].

DIAGNOSIS WITH CROSS SECTIONAL IMAGING

CT is an excellent test for cystic lesions of the pancreas because of its widespread availability and ability to detect cysts[4]. MR imaging is used increasingly because of its ability to determine if there is involvement of the main pancreatic duct[20]. Both imaging modalities offer diagnostic strengths.

CT is often the initial modality with which a cystic lesion is suspected or diagnosed, although clinical and imaging findings of chronic pancreatitis may obscure the correct diagnosis. The most common finings are a diffusely dilated pancreatic duct, cystic lesions, and parenchymal changes. The finding of a solitary septated cystic lesion in the tail of the pancreas is highly diagnostic of a mucinous cystic neoplasm (Figure 3). A grapelike cluster of involved side-branch ducts is the most common finding and represents IPMN. Thin-section contrast enhanced CT and multiplanar reconstruction may reveal communications between cystic dilated segments and the main pancreatic ducts, common findings of IPMN. Septa and excrescent nodules seen along the dilated duct have been described as features of the main pancreatic duct type in IPMT. CT is relatively poor in the diagnosis of malignant transformation. The most specific signs of malignancy with CT are a solid mass, main pancreatic duct dilatation greater than 10 mm, diffuse or multifocal involvement, and attenuating or calcified intraluminal content[21]. Serial CT imaging is often used to detect recurrence of IPMT after resection[22].

MR AND MR CHOLANGIOPANCREATOGRAPHY (MRCP)

T2-weighted MRCP images provide a detailed set of images of the main pancreatic duct and associated cystic lesions. A diffusely dilated main pancreatic duct containing mucinous filling defects is highly diagnostic of main duct IPMN. Side branch IPMN lesions appear as grape-like clusters associated with the pancreatic ductal system (Figure 4). The diagnostic accuracy using MRCP is 80% for the main duct-type and 100% for branch duct type tumors. Because of increased spatial and contrast resolution, increased sensitivity of fluid and mucin on MRCP as well as the increased sensitivity of MRI to gadolinium, the internal architecture of IPMT should be better defined with MRI and MRCP when compared to ERCP. MRCP is more sensitive than endoscopic retrograde cholangio-pancreatography (ERCP) in detecting mural nodules and wall thickening, which are of decreased signal on heavily T2 weighted MRCP sequences and are associated with malignancy.

MR imaging can reveal the full extent of ductal involvement, particularly when obstructing mucus prevents diagnostic opacification of the entire duct and is much less invasive than ERCP. MRCP findings suggestive of malignancy include: (1) filling defects/mural nodules (64% of tumors with mural nodules show invasion), (2) diffuse main pancreatic duct dilatation greater than 15 mm in main duct type. In contrast to ERCP, MRCP images the extraductal structures, and therefore is more accurate in preoperative assessment of disease. The sensitivity in the diagnosis of IPMN was highest by MRCP (88%), followed by endoscopic retrograde cholangiopancreatography (ERCP) (68%), and computed tomography scan (CT scan) (42%) and sonography (10%)[23]. Other reports

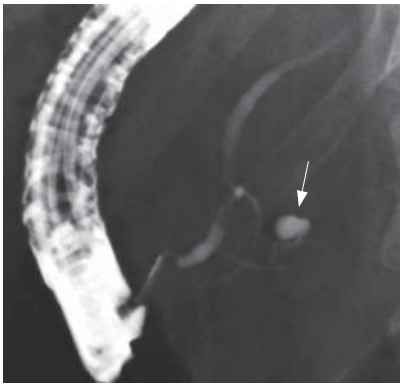


Figure 5 Pancreatogram during ERCP demonstrating a small side branch IPMN.

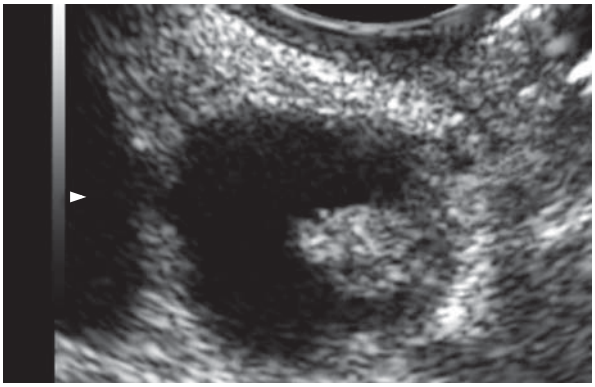


Figure 6 Linear EUS Imaging of a mucinous cystic neoplasm containing a mural nodule.

have suggested that preoperative assessment of extent using pancreatoscopy and MRCP may reduce the rate of operative positive margins and decrease postoperative recurrence. As MRCP can be performed quite rapidly without instrumentation and avoids the complications associated with ERCP, some have suggested that MRCP is superior to ERCP because it can consistently demonstrate the extent and internal architecture.

Although seen in less than 20 percent of lesions, demonstration of a central area of fibrosis by CT or MR is a highly diagnostic feature of a serous cystadenoma. The honey-combed or microcystic appearance of the lesion is commonly used to provide a diagnosis. Mucinous cystic neoplasms, in contrast, are commonly diagnosed with CT based on the unilocular or macrocystic characteristics. Although not frequently seen, the finding of peripheral calcification of the wall is specific for a mucinous cystic neoplasm. Intraductal papillary mucinous neoplasms (IPMN) may involve the main pancreatic duct exclusively, a side-branch or both. MRCP can demonstrate the diagnostic findings of pancreatic duct dilation, mural nodules, and ductal connection better than ERCP.

Despite these imaging features, the ability to accurately diagnose a specific cystic lesion and to determine whether malignancy is present by CT and MR remains uncertain. The diagnosis of a pancreatic pseudocyst is more dependent upon the clinical history and the associated findings of chronic pancreatitis. Pancreatic pseudocysts appear as unilocular fluid-filled cavities associated with parenchymal changes such as calcifications and atrophy.

THE ROLE OF ENDOSCOPY

An endoscopic evaluation may be obtained when cross sectional imaging has detected a cystic lesion, but no diagnostic features are apparent[24]. In selected patients, endoscopy is used to provide the cause of recurrent pancreatitis[3]. ERCP and EUS are powerful tools in the diagnosis of cystic and intraductal neoplasms. ERCP excels at the detection of main duct IPMNs and can provide cytologic material from strictures, nodules, or intraductal masses (Figure 5). Side branch IPMN lesions appear as fluid-filled dilated ductal branches filled with mucin (Figure 6). The finding of a focal pancreatic duct stricture in the setting of a cystic lesion is highly suggestive of a malignant process[25].

Pancreatography and pancreatoscopy are very sensitive techniques for the diagnosis of IPMNs[26]. The diagnostic features of IPMN include frond-like epithelial protuberances that are active in the secretion of intraductal mucin[26]. During ERCP, diagnostic tissue can be obtained from the ductal epithelium and analyzed using cytology as well as molecular markers[27]. Intraductal pancreatoscopy can be supplemented with the use of intraductal ultrasound to detect early malignancies arising from IPMN lesions[28]. 88% of fish-egg- like villous masses protruding more than 4 mm were found to be malignant.

The disadvantages of ERCP for the evaluation of patients with relapsing pancreatitis and suspected cystic neoplasm include the risk pancreatitis, poor quality of epithelial samples, and the technical difficulties of placement of a small probe through the pancreatic duct and sphincter. Many of the challenges will be overcome in the near future when small diameter high resolution digital probes are released for use.

ROLE OF EUS

EUS is often performed in the evaluation of relapsing pancreatitis when cross sectional imaging has not provided a definitive diagnosis. In nearly 60% of patients, EUS can provide a cause of recurrent pancreatitis[29]. The most common EUS finding in patients with recurrent pancreatitis is parenchymal changes suggestive of chronic pancreatitis[30]. EUS findings were similar in those with a single episode of idiopathic pancreatitis *vs* those with recurrent episodes. There is now some evidence in the literature suggesting that these early changes detected by EUS correlate with the histological changes of chronic pancreatitis and may predict progression to more advanced disease[31]. The EUS diagnosis of chronic pancreatitis relies on quantitative parenchymal and ductal criteria. It is generally accepted that, in the absence of any criteria, chronic pancreatitis is unlikely, whereas in the presence of 5 or more criteria (out of 9-11) chronic pancreatitis is likely. Diffuse changes may also represent autoimmune pancreatitis[32].

IPMN of the pancreas is the most common malignancy associated with recurrent pancreatitis[33]. Although there is considerable overlap between the parenchymal features of IPMN and chronic pancreatitis, EUS is fairly reliable in

Table 1 Characteristics of cystic neoplasms

	Morphology	Location	Ductal communication	Associated with pancreatitis	Cyst fluid CEA (pg/mL)
Serous	Microcystic	Evenly distributed	No	Rare	< 0.5
MCN	Macrocystic	Tail	Rare	Occasionally	> 200
IPMT	Mixed	Head	Yes	Yes	> 200
Pseudocyst	Unilocular	Evenly distributed	Yes	Yes	< 200

differentiating between IPMN and CP[33]. When compared with patients with chronic pancreatitis, the EUS features of dilation of pancreatic duct, cysts, and pancreatic atrophy were the most common findings of IPMN. The majority of IPMNs at the time of diagnosis are non-invasive and benign[34]. In a small percentage of patients, EUS will demonstrate a focal malignancy[29]. Pancreatic malignancies may arise from IPMN or from remote sites within the pancreas[35].

Mucinous cystic neoplasms (MCN) are a rare cause of recurrent pancreatitis. Nevertheless, these solitary cystic lesions may be encountered during EUS (Figure 6). Morphologically, these mucinous lesions are usually thin-walled and contain a few thin septations. FNA reveals thick, translucent fluid characterized by a mucinous epithelium. Cyst fluid CEA > 200 pg/mL is the most diagnostic parameter[36] (Table 1).

Serous cystadenomas, just like MCNs, rarely communicate with the main pancreatic duct and therefore rarely cause pancreatitis. Morphologically, the EUS appearance of serous cystadenoma is unique. The multiple fine septations coursing across the cyst result in a microcystic or a honeycomb appearance. At times, the septations are so dense that the lesion appears as a solid mass. The increased vascularity of the lesion, driven by high levels of vascular endothelial growth factor (VEGF) results in bloody contamination of cyst fluid during FNA.

TREATMENT

Pre-malignant cystic lesions should be resected surgically unless the risks of surgery are excessive. The decision to resect a lesion, however, is based on the presence or absence of symptoms, the risk of malignancy, and the surgical risk of the patient. High risk patients with low grade cystic neoplasms may be monitored with periodic CT/MRI scanning or EUS-FNA. Small cystic lesions in the elderly can be safely monitored.

The increasing safety of surgical resection has encouraged the use of surgical resection for a wider range of lesions (Table 2). However, many cystadenomas do not require resection except for relief of symptoms. Furthermore, mucinous lesions are slow growing lesions and small lesions pose little risk. The risk of surgical resection, particularly of the head has raised questions about management[37]. Justification of surgery is readily obtained with high risk lesions as defined by cyst fluid analysis[38]. Since most mucinous cystic neoplasms are located in the tail of the pancreas, a distal pancreatectomy is sufficient for these pre-malignant lesions. Lesions in the body of pancreas can be selectively resected with a middle segmentectomy. The highest risk surgical approach is for lesions in the head of the pancreas.

Table 2 Traditional therapeutic approach to the management of cystic lesions

Location	Mucinous	Malignant	Serous	Pseudocyst
Head	Monitor	Resect	Monitor	Drain
Body	Resect	Resect	Monitor	Drain
Tail	Resect[1]	Resect	Resect[1]	Resect

[1]Approach varies with risk of surgery.

CONCLUSION

Patients with relapsing pancreatitis associated with a cystic lesion pose an important challenge to the clinician. A diagnosis of a cystic neoplasm is very important because this finding will provide an opportunity to resect a lesion responsible for pancreatitis and a risk of malignancy. An accurate diagnosis requires a thorough clinical evaluation combined with high resolution pancreatic imaging. Cyst fluid analysis will often complement and strengthen the diagnosis of a cystic neoplasm and provide the basis for a surgical resection.

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ESOPHAGEAL CANCER

Overexpression of Slug is associated with malignant progression of esophageal adenocarcinoma

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Abstract

AIM: To characterise expression of known *E-cadherin* repressors; *Snail*, *Slug* and *Twist* in the development of esophageal adenocarcinoma.

METHODS: *E-cadherin*, *Slug*, *Snail* and *Twist* mRNA expression in Barrett's metaplasia and esophageal adenocarcinoma specimens was examined by real-time reverse transcription-polymerase chain reaction (RT-PCR). Semi-quantitative immunohistochemistry was used to examine cellular localisation and protein levels. The effect of *Slug* on epithelial mesenchymal transition (EMT) markers was examined by transfection of Slug into an adenocarcinoma line OE33.

RESULTS: Cellular localisation of Slug in Barrett's metaplasia was largely cytoplasmic whilst in adenocarcinoma it was nuclear. Semi-quantitative analysis indicated that *Slug* was more abundant in adenocarcinoma compared to matched Barrett's metaplastic specimens. Snail and Twist were expressed in adenocarcinoma but were cytoplasmic in location and not induced compared to Barrett's mucosa. These observations were supported by mRNA studies where only *Slug* mRNA was shown to be over-expressed in adenocarcinoma and inversely correlated to E-cadherin expression. Overexpression of *Slug* in OE33 mediated *E-cadherin* repression and induced the mesenchymal markers vimentin and fibronectin.

CONCLUSION: Progression to adenocarcinoma is associated with increased Slug expression and this may represent a mechanism of *E-cadherin* silencing.

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Key words: Slug; Oesophagus; Cancer; Barrett's metaplasia; Epithelial-mesenchymal transition

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INTRODUCTION

The incidence of esophageal adenocarcinoma is currently rising faster than any other cancer in the Western world. Alarmingly the cause of this increase is largely unknown[1]. The strongest known risk factor for esophageal adenocarcinoma is the condition Barrett's metaplasia which is characterised by a replacement of the native squamous lined esophagus with a columnar epithelium: a possible consequence of prolonged reflux of gastric contents into the lower esophagus[1,2].

To date, numerous proteins have been implicated in the malignant progression of Barrett's metaplasia to adenocarcinoma including E-cadherin[3-6]. E-cadherin is a calcium dependent cell adhesion molecule which is essential in the establishment of homotypic adhesion junctions[7]. In nearly all epithelial cancers E-cadherin has been reported to be repressed: an essential step for increased invasiveness and metastasis[8-10], thus this event commonly occurs during late tumourigenesis. In particular in the development of esophageal adenocarcinoma, E-cadherin repression has been reported to be a late event associated with high grade dysplastic Barrett's metaplasia

and adenocarcinoma[4-6].

However, it remains unclear what directs repression of E-cadherin expression in esophageal adenocarcinoma. Unlike in hereditary diffuse gastric cancer *E-cadherin* is not subject to mutation and neither is there evidence of *E-cadherin* promoter methylation as is common in colorectal cancers[11,12]. A possible mechanism of *E-cadherin* silencing, which to date has not been addressed, is transcriptional repression by proteins involved in epithelial mesenchymal transition (EMT) including proteins in the Snail family: Snail and Slug and the transcription factor *Twist*[13-15].

Several studies have elegantly demonstrated in a variety of cell types that overexpression of Snail and Slug causes an EMT and this is associated with E-cadherin repression[16,17]. Furthermore, it has been demonstrated that these effects are mediated through interaction with specific *E-pal* elements of the *E-cadherin* proximal promoter[16,17]. In support of a role for these EMT regulators in E-cadherin repression and carcinogenesis, several studies have shown overexpression in several epithelial cancers including overexpression of Slug in gastric carcinomas[18], Snail in colorectal cancers[19] and Twist in pancreatic cancers[20].

The primary aim of this study was to determine the expression profile of Snail, Slug and Twist in the development of esophageal adenocarcinoma and determine if overexpression of these proteins in an esophageal background is able to mediate a repression in E-cadherin.

MATERIALS AND METHODS

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 University Hospital Birmingham Trust (LREC 2002/166). All patients provided informed written consent.

Patient tissue

Esophageal adenocarcinoma resection specimens: Samples of normal squamous esophagus (n = 40) and esophageal adenocarcinoma (n = 40) were collected during surgery. Half of the esophageal adenocarcinoma resection specimens (n = 20) collected also had associated intestinal Barrett's metaplasia. In addition, samples (n = 20) of long segment ($\geqslant$ 3 cm) Barrett's metaplasia, defined as columnar mucosa with intestinal type goblet cells were also collected during endoscopy. All specimens were divided in two, half for RNA extraction and half for immunohistochemistry.

Immunohistochemistry: Immunohistochemistry for E-cadherin was performed using microwave antigen retrieval as previously described with an E-cadherin monoclonal antibody (clone 36, BD Biosciences, Oxford, UK) used at a concentration of 1:300[21]. Immunohistochemistry for Snail, Slug and Twist was performed as follows: Slides were immersed in W-cap buffer (Bio-Optica, Milan, Italy) and cycled in a Pixel antigen retriever (CellPath, Newtown, UK) for 60 min, washed in running water and placed in methanol:hydrogen peroxide (10:1) for 5 min. Sections were then incubated in a primary antibody to either Snail, 1:10, (SNAI1 clone E18, Autogen Bioclear, Wiltshire, UK), Slug, 1:20 (SNAI2 clone G18 Autogen Bioclear, UK) or Twist, 1:50 (clone C17 Autogen Bioclear, UK) in TBS 7.5 × buffer (Bios Europe Ltd, Skelmersdale, UK) at 4℃ overnight, washed with TBS and reacted with peroxidase-linked rabbit anti-sheep antibody (Dako, Ely, UK) at a 1:100 dilution in TBS for 1 h. The immunoreactivity was then revealed as above using DAB. Slides were then dipped in hematoxylin, dehydrated and mounted. The slides were scored by a previously described method for (1) intensity of staining (0 = negative, 1 = weak, 2 = moderate, 3 = intense) and (2) percentage of epithelial cells staining (0 = 0%-5%; 1 = 6%-25%; 2 = 26%-50%; 3 = 51%-75%; 4 = 76%-100%); these two scores were multiplied to yield a final staining score[39]. In addition, cellular localisation (nuclear, cytoplasmic, cell surface) was assessed. All sections were scored independently by two observers (PJ and CT).

In the series of immunofluorescent experiments following primary antibody incubations, sections were washed extensively and then incubated with either FITC goat anti-mouse or goat anti-rabbit (Jackson Immunoresearch, USA, 1:500) for 1 h. Sections were then washed and incubated in 4', 6-Diamidino-3-phenylindole dihydrochloride hydrate (DAPI) (1:10 000) for 1 min prior to visualisation. Omission of primary antibody was employed as a negative control. Images were visualised using an Olympus BX40 microscope and digital images taken using a Sensys Photometrics camera (Middlesex, UK). Desksoft SmartCapture 2 software was used for image acquisition (Desksoft, USA).

Real time RT-PCR

Real time RT-PCR reactions were performed as previously described using 18S ribosomal RNA as an internal standard (PE Biosystems, Roche, USA)[21]. Each reaction was performed in triplicate and contained one of the following sets of probes and primers: (1) *E-cadherin* (probe 5'FAM AAATTCACTCTGCCCAGGACGCGGTAMRA3'), forward primer (5'-GGCGCCACCTCGAGAGA-3') and reverse primer (5'-TGTCGACCGGTGCAATCTT3'); (2) *Slug* (probe 5'FAM'CACATACAGTGATTATTTC CC CGTATCTCTATGAGAGTTAMRA3'), forward primer (5'-AAAAGCCAAACTACAGCGAACTG-3') and reverse primer (5'-AGAATCTCTGCTTGTGGT ATG ACA-3'); (3) *Snail* (probe 5'FAMTGCAGGACTCTA ATCCAGAGTTTACCTTCCAGCTAMRA3'), forward primer (5'-CCCAATCGGAAGCCTAACTACAG-3') and reverse primer (5'-CAGGTGGGCCTGGTCGTA-3'), or (4) *Twist* (probe 5'-FAMACAATGACATCTAGG TCTC-3'), forward primer (5'-GCTCCAGAGTCTCTA GACTGTCCATT-3') and reverse primer(5'-GGGGCCTGGTCCATGTC-3').

Western blotting

Western blotting was performed as previously described[21] with antibodies to (1) E-cadherin (1:2 000, clone 36, Autogen Bioclear UK); (2) Fibronectin (1:1000, Clone

P1F11 Autogen Bioclear, UK); (3) Vimentin (1:1000, clone C-20, Autogen Bioclear, UK); (4) Cytokeratin 19 (CK-19) (1:2000; Oncogene Research Products USA) and (5) GFP (1:2000, Clone AB290 Abcam, UK). Immunoreactive bands were then subject to densitometry using NIH Image 1.62 software.

Cell culture

Cell lines derived from esophageal adenocarcinoma (OE33[22], SEG-1[23] and oesophageal squamous carcinoma TE-7[24]) were all routinely cultured in Dulbecco's modified Eagles medium (Gibco, USA) with 10% fetal calf serum supplemented with 1×10^5 Units/L penicillin and 1 g/L streptomycin. Upon reaching 70% confluence cells were lysed into Trizol reagent (Gibco, UK) for mRNA extraction and evaluation of *Slug* mRNA expression by Real Time PCR.

Transfection studies

Cell line OE33 was transiently transfected with either control empty vector, pMSCV-GFP or full-length human *Slug*-GFP tagged construct, pMSCV-*Slug*-GFP[25] or beta-galactosidase plasmid (beta-GAL) using Lipofectamine Plus according to manufacturers instruction. 48 h post transfection cells were either assayed for transfection efficiency using beta-galactosidase assay to determine transfection efficiency, lysed into RIPA buffer for Western blotting or lysed in Trizol for real time RT-PCR studies. Data analysis was only performed in experiments where a minimum of 40% transfection efficiency was achieved as determined by beta-galactosidase assay.

To determine the effect of *Slug* on the human *E-cadherin* promoter, an *E-cadherin* promoter assay was performed as previously described[21]. Briefly, OE33 cells were transiently transfected with full length human *slug* (pCDNA3-*Slug*[25]), *Renilla* luciferase plasmid pRL TK and the wild-type human *E-cadherin* promoter cloned into the pGL3basic luciferase reporter plasmid (EproWT) a kind gift from Professor Frans van Roy). Controls included empty pCDNA3 and empty pGL3basic vectors. 48 h post transfection cells were assayed for firefly and *Renilla* luciferase activities using the dual-reporter assay kit Stop 'N' Glow (Promega) according to manufacturer's instruction. Firefly luciferase activity was normalised to *Renilla* luciferase activity as a transfection control. Promoter activity was expressed as a fold change in relative luciferase units (RLU) compared to that obtained in pGL3basic control transfected cells. Results shown represent the means ± SE of three independent experiments.

Statistical analysis

All data are presented as means ± SE. Statistical significance was calculated using unpaired Student's *t* test. To assess the association between *Slug* and *E-cadherin*, linear regression analysis was performed on log-transformed mRNA fold change values using log *Slug* expression as the independent variable and log *E-cadherin* expression as the dependent variable. The R^2 value was determined to demonstrate the extent to which expression of *Slug* was correlated to *E-cadherin*. A Bonferroni adjustment was applied to *P* values attained from multiple comparisons during the analysis of prognostic factors. In univariate analyses significance was accepted at $P \leqslant 0.05$, following Bonferroni adjustment, $P \leqslant 0.0125$ was considered significant. All analyses were performed using SPSS version 10.0 (SPSS Inc, USA).

Table 1 Semi-quantitative analysis of Snail, Slug and Twist in malignant progression of Barrett's metaplasia to adenocarcinoma (Immunoreactivity score)

	S	BM (-ve)	BM (+ve)	ADC
Snail	2.4	2.5	2.9	8.7[a]
Slug	6.7	2.6	3.5	10.5[a,c]
Twist	4.5	4.7	5.2	7.1

Semi-quantitative analysis of immunoreactivity for Snail, Slug and Twist in specimens of squamous esophagus (S); Intestinal type Barrett's metaplasia without associated adenocarcinoma BM (-ve), intestinal Barrett's metaplasia with associated adenocarcinoma BM (+ve) and esophageal adenocarcinoma (ADC). The mean staining scores were calculated for each type of mucosa and the mean fold change in protein expression was calculated. [a]denotes significant ($P < 0.05$) differences between adenocarcinoma and intestinal type Barrett's metaplasia without associated adenocarcinoma. [c]denotes significant ($P < 0.05$) differences between adenocarcinoma and intestinal type Barrett's metaplasia with associated adenocarcinoma.

RESULTS

Immunolocalisation of Snail, Slug and Twist in esophageal adenocarcinoma

In normal stratified squamous esophagus, there was no detectable expression of Snail and only weak diffuse cytoplasmic immunoreactivity for Twist, while there was strong nuclear immunoreactivity for Slug in basal and supra-basal layers (Figure 1). In Barrett's metaplasia, Snail and Slug were generally localised to the cytoplasm with staining being patchy and diffuse in nature. In contrast, Twist was largely nuclear in localisation (Figure 1). In esophageal adenocarcinoma, all three proteins were abundantly expressed with Snail and Twist being mostly localised in the cytoplasm whilst Slug was almost exclusively nuclear in all adenocarcinoma specimens ($n = 20$) (Figure 1).

In addition, all slides were scored and data are shown in Table 1. To determine if Snail, Slug and Twist were over-expressed in adenocarcinoma specimens relative to non-matched Barrett's metaplastic specimens (BM-ve), statistical analysis was performed and results showed that both Slug and Snail were significantly over-expressed ($P < 0.05$) in esophageal adenocarcinoma. Twist, however, was not significantly induced. To validate this data, we further performed immunohistochemistry on a further 20 sections of adenocarcinoma with matched Barrett's metaplastic tissue (BM+ve). When comparing the staining scores of matched Barrett's metaplastic specimens with adenocarcinoma, only Slug was significantly over-expressed ($P < 0.05$).

Since there is a growing body of evidence implicating Slug as a repressor of E-cadherin, we further sought to address if Slug overexpression was associated with depressed E-cadherin expression in sections of both Barrett's metaplasia and adenocarcinoma using dual immunofluorescence with anti-Slug (FITC) and E-cadherin antibodies (Texas Red) (Figure 2). Sections of Barrett's

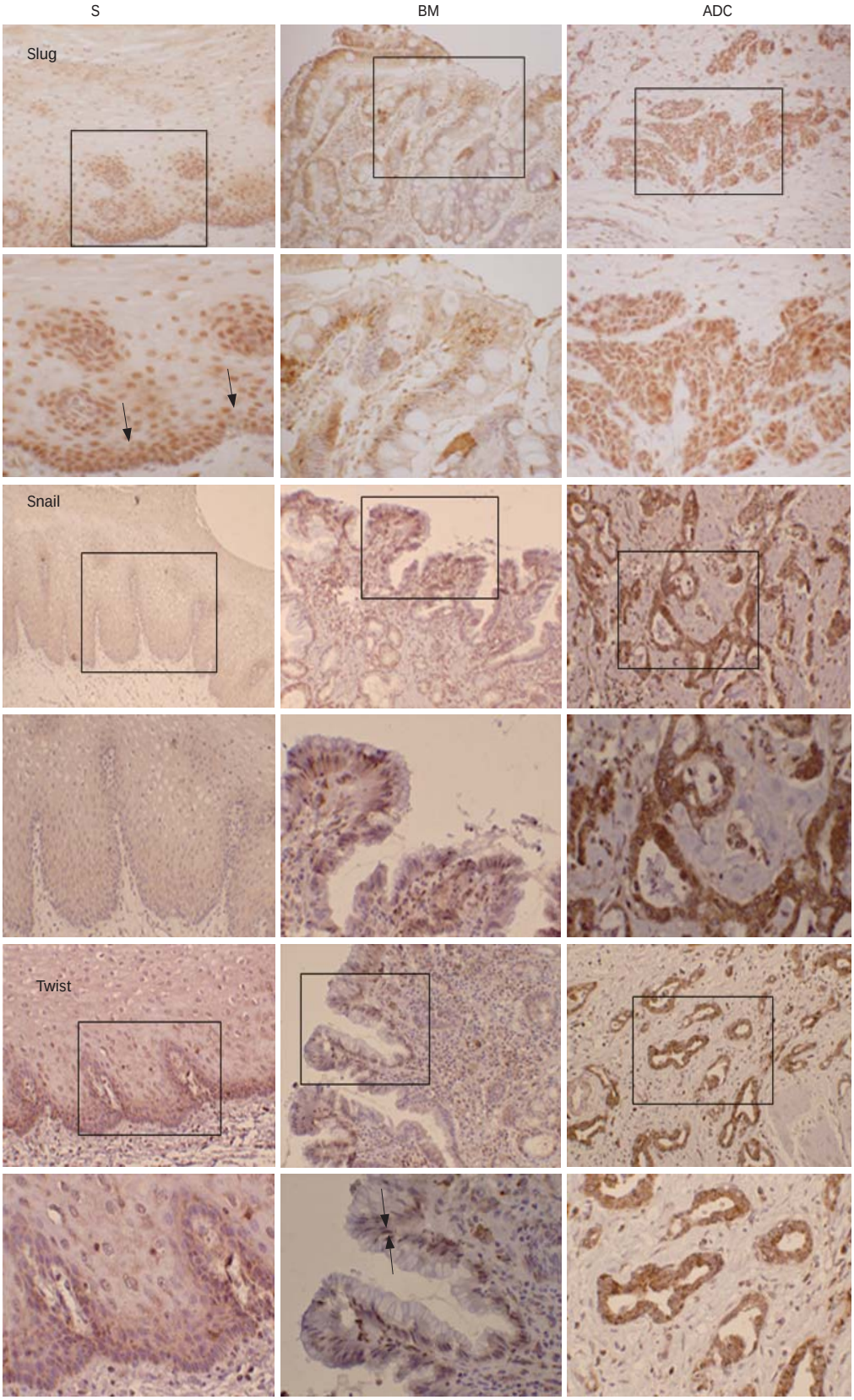


Figure 1 Immunolocalisation of Slug, Snail and Twist in normal esophagus, intestinal type Barrett's metaplasia and oesophageal adenocarcinoma. Normal squamous esophagus (S), intestinal type Barrett's metaplasia (BM) and esophageal adenocarcinoma (ADC) were subjected to immunohistochemistry using antibodies to Slug, Snail and Twist. Arrows denote areas of positivity (× 20, × 40).

metaplasia mostly demonstrated preserved cell border E-cadherin immunoreactivity consistent with its role in cell-cell adhesion whilst there was little detectable expression of Slug. Conversely in sections of adenocarcinoma in areas of abundant Slug immunoreactivity, there was almost a complete loss of E-cadherin immunoreactivity with the expression mostly localised to the cytoplasm.

mRNA expression of E-cadherin, Slug, Snail and Twist in Barrett's metaplasia and esophageal adenocarcinoma specimens

To determine if *Slug*, *Snail*, *Twist* and *E-cadherin* were modulated at the transcriptional level in esophageal adenocarcinoma, 10 specimens of esophageal adenocarcinoma each with matched intestinal Barrett's

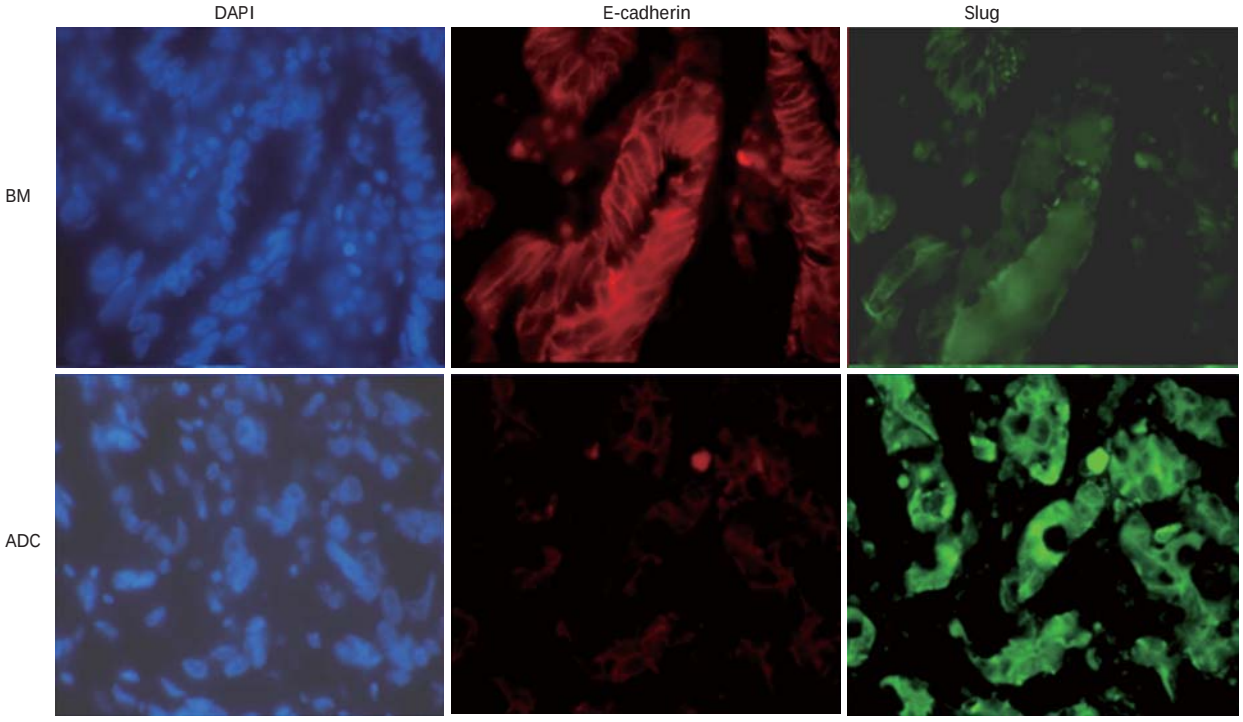


Figure 2 Co-immunofluoresence of Slug and E-cadherin in Barrett's metaplasia and esophageal adenocarcinoma. Sections of Barrett's metaplasia (BM) mostly demonstrate preserved cell border E-cadherin (Texas Red) immunoreactivity consistent with its role in cell-cell adhesion. Slug (FITC) immunoreactivity in BM however was minimal and appeared non specific. Conversely in sections of adenocarcinoma (ADC) in areas of abundant Slug immunoreactivity, there was negligible E-cadherin immunoreactivity. DAPI staining was used to highlight nuclei in sections ($\times$ 40).

metaplasia were analysed by real-time PCR (Figure 3).

E-cadherin mRNA was repressed in 70% of adenocarcinoma specimens whilst *Slug*, *Snail* and *Twist* was over-expressed in 70%, 40% and 50%, respectively. The mean fold decrease for *E-cadherin* in the ten specimens examined was 0.73 whilst *Slug*, *Snail* and *Twist* had mean fold increases of 2.07, 1.47 and 1.50 respectively. We then sought to determine if the mean fold changes for *Slug*, *Snail* and *Twist* across the ten specimens were significantly different from *E-cadherin* values. This revealed that only *Slug* was significantly elevated compared to *E-cadherin* expression ($P < 0.005$). Additionally we assessed the association between *E-cadherin* and these EMT regulators using linear regression analysis. Results of this showed that again only *Slug* was negatively correlated with *E-cadherin* expression (R^2 0.677, $P < 0.03$) (Figure 4).

Exogenous overexpression of Slug in the cell line OE33 induces an EMT phenotype

Slug mRNA expression was examined in a panel of three esophageal cell lines OE33, SEG1 and TE-7 by real-time PCR and results showed that the cell line OE33 had the lowest expression of *Slug* mRNA whilst the highest *Slug* expressing cell line was TE-7. In this regard, the cell line OE33 was chosen for *Slug* overexpression studies.

The cell line OE33 was transiently transfected with either full length human *Slug*-GFP vector or the control empty GFP vector. Forty-eight h after transfection, cells were either lysed and processed for mRNA and protein analysis or fixed for immunofluorescence.

mRNA analysis revealed an approximate 6.5 $\times$ induction in *Slug* mRNA in cells transfected with full length human *Slug*-GFP compared to cells transfected with empty-GFP vector ($P < 0.05$) (Figure 5A). Exogenous *Slug* expression was further verified indirectly (due to a lack of a commercially available *Slug* antibody suitable for Western blotting) by Western blotting for GFP (Figure 5B).

To ascertain whether overexpression of *Slug* in OE33 cells mediated an EMT phenotype, Western blotting was performed to examine protein expression of three EMT markers *E-cadherin*, vimentin and fibronectin (Figure 6). Densitometric analysis of Western blots ($n = 6$) revealed *Slug* overexpression was associated with a repression in *E-cadherin* (mean fold decrease of 53% $\pm$ 15%, $P < 0.05$) and increased vimentin (41% $\pm$ 11%, $P < 0.05$) and fibronectin expression (32% $\pm$ 12%, $P < 0.05$). In addition, the cell adhesion and signalling molecule beta-catenin was also examined and shown to be significantly repressed (57% $\pm$ 19%, $P < 0.05$).

To address whether *Slug* mediated *E-cadherin* protein repression was through a direct effect at the level of the *E-cadherin* promoter and consequently a transcriptional effect, a luciferase *E-cadherin* promoter assay and real-time PCR was employed respectively as previously described[21,25]. Overexpression of *Slug* in OE33 resulted in a significant reduction (35% $\pm$ 10%, $P < 0.05$) in *E-cadherin* promoter activity (Figure 7A) which was mirrored by decreased *E-cadherin* mRNA expression (20% $\pm$ 5%, $P < 0.05$) (Figure 7B).

DISCUSSION

Recent reports have highlighted the importance of

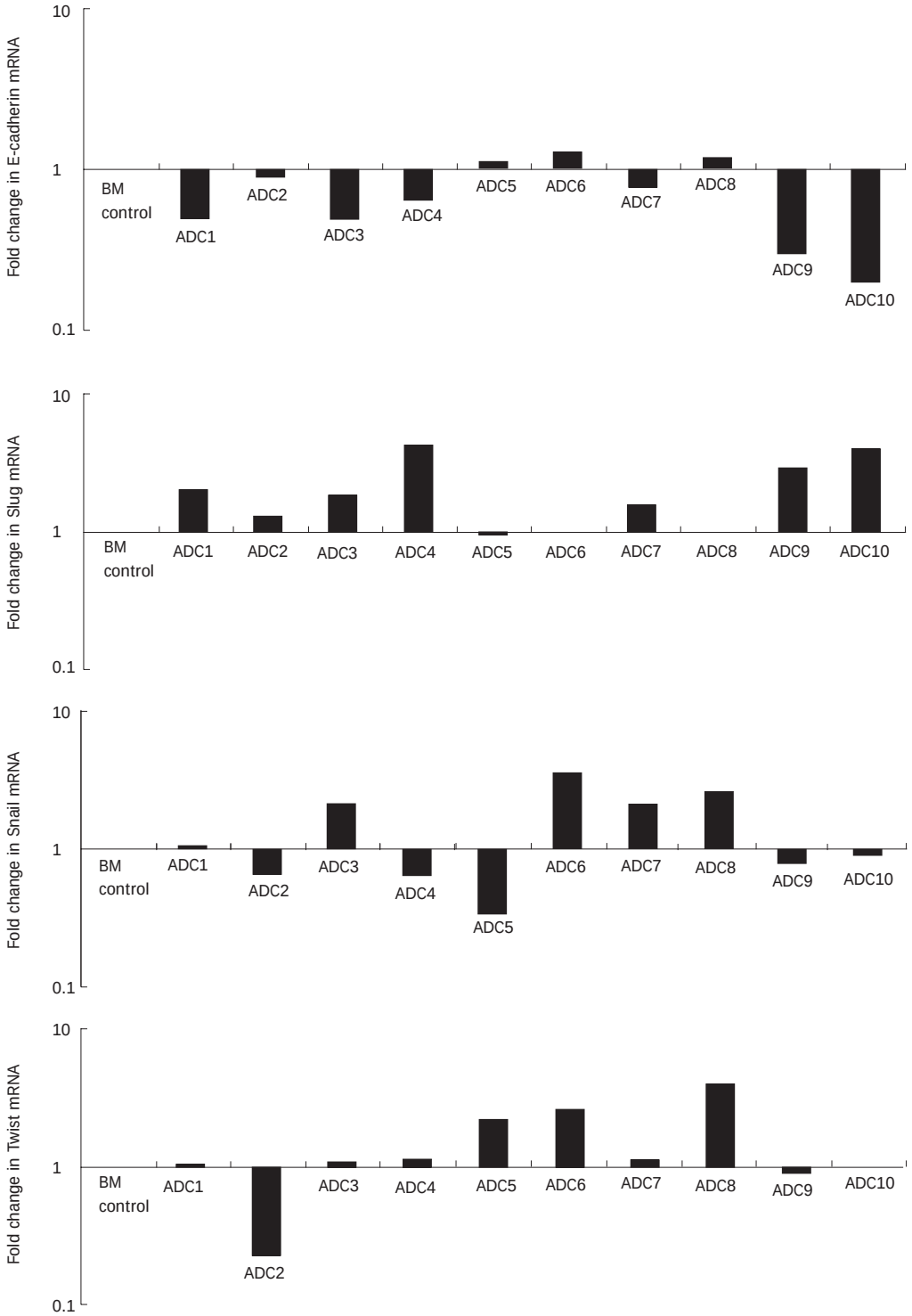


Figure 3 mRNA expression of *E-cadherin*, *Slug*, *Snail* and *Twist* in esophageal adenocarcinoma. Real time RT-PCR was employed to examine the expression of *E-cadherin*, *Slug*, *Snail* and *Twist* in ten esophageal adenocarcinoma specimens (ADC1-ADC10) each compared to Barrett's metaplastic tissue collected from the same resection specimen. Barrett's metaplastic tissue control (BM), normalised to 1.0.

epithelial mesenchymal regulators including Snail, Slug and Twist in gastrointestinal carcinogenesis[18,19,26-28]. We have demonstrated for the first time a modulation in the expression and localisation of these proteins in the malignant progression of normal squamous esophagus to adenocarcinoma.

Twist was only weakly expressed in squamous esophagus consistent with the results of Yuen *et al*[28]. Their studies reported over 95% of non-neoplastic squamous esophageal samples were either negative or weak for Twist immunoreactivity as assessed by immunohistochemistry. However, in our studies we did observe nuclear Twist staining in Barrett's metaplasia which in adenocarcinoma had become cytoplasmic in localisation. This pattern of cytoplasmic expression for Twist in cancer has also been observed in squamous cell cancers of the esophagus[28]. We were unable to show a difference in Twist expression between matched Barrett's and adenocarcinoma specimens

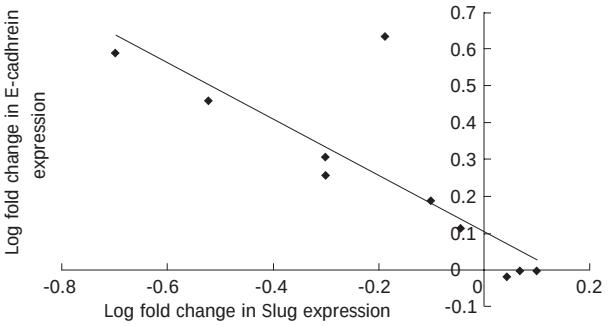


Figure 4 Correlation of *E-cadherin* and *Slug* mRNA expression in esophageal adenocarcinoma specimens. Using linear regression analysis, *Slug* mRNA expression was demonstrated to be negatively correlated with *E-cadherin* expression in the ten adenocarcinoma specimens examined (ADC1-10) (R^2 0.677, $P < 0.03$).

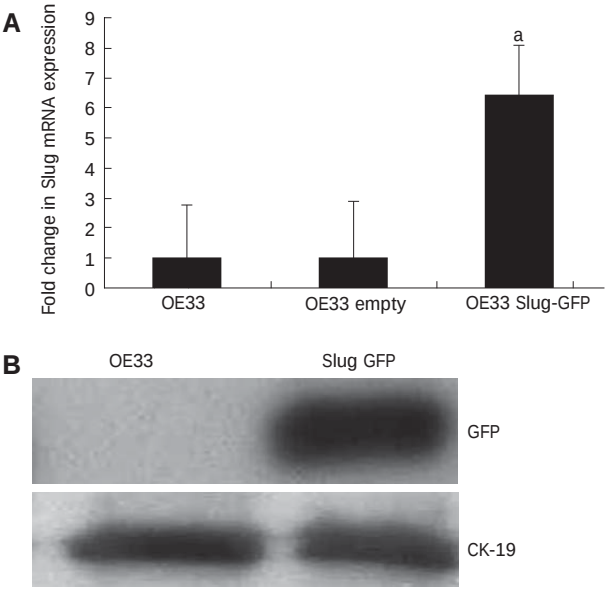


Figure 5 Overexpression of *Slug* in the esophageal adenocarcinoma derived cell line OE33. Cell line OE33 was transiently transfected with either full length human *Slug*-GFP vector (OE33 *Slug*-GFP) or with the control empty GFP vector (OE33 empty). 24 h post transfection, (**A**) *Slug* mRNA and (**B**) GFP protein expression was determined. CK-19 was used as an epithelial control. $^aP < 0.05$ using Student's *t* test.

at both the mRNA and protein level, and neither was its expression associated with prognostic end points such as stage and modal involvement.

In the case of Snail, this was comparably either very weak or nondetectable in stratified squamous oesophagus whilst in both Barrett's metaplastic tissue and adenocarcinoma Snail was detectable in the cell cytoplasm. Despite moderate expression of Snail in adenocarcinoma specimens, there was no difference in expression between matched adenocarcinoma and Barrett's specimens at both the protein and mRNA level. A recent study has also reported Snail immunoreactivity in adenocarcinomas of the upper gastrointestinal tract including the esophagus[29]. In their study Snail expression was reported in 11% of esophageal adenocarcinomas examined and no correlation was found between the expression of Snail and tumor grade and stage.

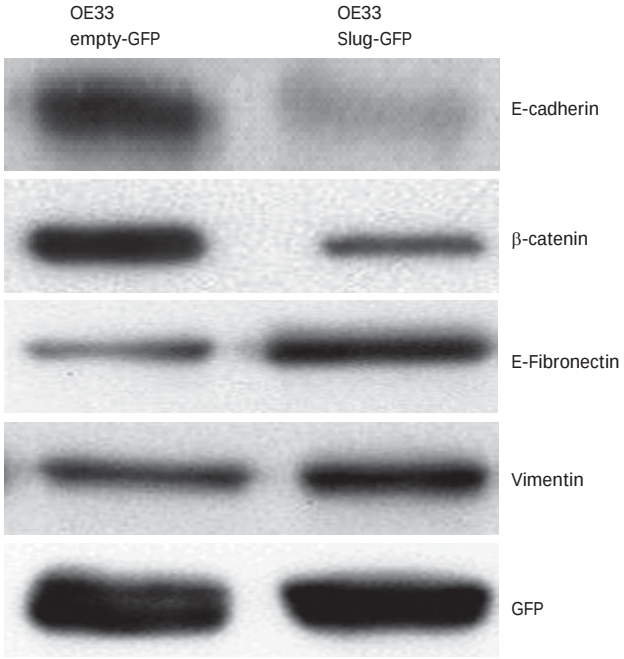


Figure 6 The effect of Slug overexpression on proteins implicated in EMT. Expression of E-cadherin, beta-catenin, fibronectin and vimentin were all examined in Slug over-expressing (OE33 Slug-GFP) and control cells (OE33 empty-GFP). Lysates were normalised using GFP expression.

Why both Snail and Twist are localised to the cytoplasm in esophageal adenocarcinoma is unclear though a recent study has suggested that Snail's localisation could in part be determined by post-translational phosphorylation with proteins such as p21-activated kinase 1 (Pak1)[30]. Interestingly, when Pak1 mediated phosphorylation of Snail was inhibited, this not only caused a nuclear to cytoplasmic accumulation but also attenuated its repressor activity[30].

Immunolocalisation studies for the transcription factor Slug showed that it was strongly expressed in nuclei of basal and suprabasal esophageal keratinocytes of the stratified squamous epithelium consistent with previous reports in murine studies[31] and other human stratified epithelia including epidermis[25]. Whilst Slug nuclear expression was lost in Barrett's metaplasia, it was retained in adenocarcinoma and furthermore there appeared to be an overexpression of this protein in adenocarcinoma compared to matched Barrett's metaplasia at both the mRNA and protein level.

Interestingly Uchikado *et al* have reported that Slug is also over-expressed in esophageal squamous cell carcinomas (SCC)[27]. Their study showed that the presence of Slug was associated with depth of tumor invasion, lymph node metastasis, stage and lymphatic invasion. Consistent with our study, Slug expression in SCC was significantly correlated with reduced E-cadherin expression.

Thus our data would suggest that of the three EMT regulators examined, Slug was the only transcription factor to show a significant increase at both the mRNA and protein level in the progression from Barrett's metaplasia to adenocarcinoma. This coupled with nuclear expression in adenocarcinoma and an association with E-cadherin repression suggests functionality in oesophageal tumourigenesis.

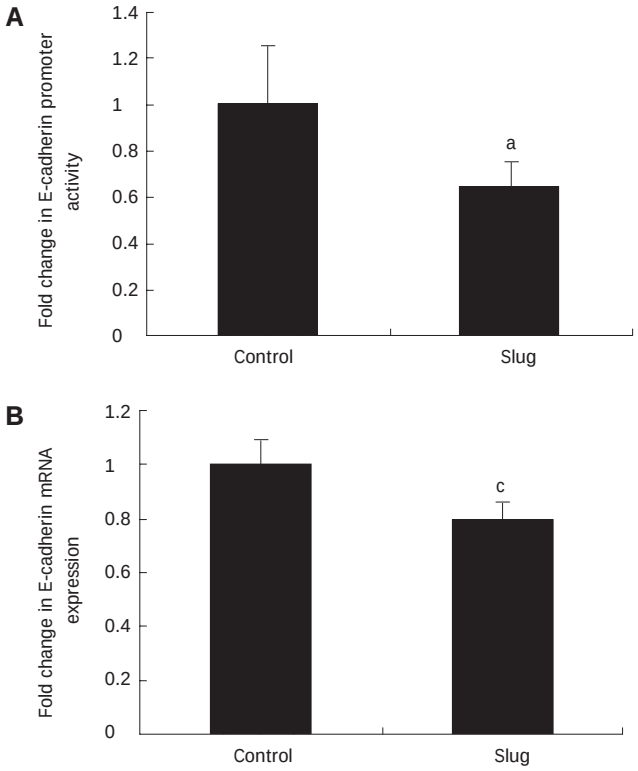


Figure 7 *Slug* expression causes a decrease in *E-cadherin* promoter activity and mRNA expression. **A**: OE33 cells transfected with full length human *Slug* (*Slug*) showed decreased *E-cadherin* promoter activity compared to sham transfected control cells. Promoter activity was expressed as a fold change in relative luciferase units (RLU) compared to control cells. Results represent the means ± SE of three independent experiments. $^{a}P < 0.05$ using Student's *t* test; **B**: Expression of *E-cadherin* mRNA in *Slug* over-expressing cells (*Slug*) was also repressed in comparison to control cells (control). Relative gene expression was normalised to 1.0 (100%) of controls. Error bars = 2 mean ± SE. $^{c}P < 0.05$ using Student's *t* test. These data are the mean of three independent experiments.

The consequence of overexpression of Slug in adenocarcinoma is likely to be complex, as exemplified by the ever-growing list of direct and indirect downstream target genes attributed to be modulated as a consequence of its expression[25,32,33]. As anticipated, many of these targets appear to be involved in cellular survival, proliferation and mesenchymal transition. The latter was exemplified by E-cadherin, a protein commonly silenced in epithelial cancers including esophageal adenocarcinoma[4-6,8-10,17].

To support our hypothesis that *Slug* might be responsible for the observed repression of *E-cadherin* in adenocarcinoma, Slug was exogenously over- expressed in an oesophageal cell line OE33. Our results demonstrate that *Slug* could repress *E-cadherin* transcription at the level of the *E-cadherin* promoter. *Slug* mediated *E-cadherin* promoter repression has been previously demonstrated in other lineages and this is likely to be mediated through one or more of the *E-box* elements present within the promoter[17].

To verify the extent of EMT, we further examined the expression of the mesenchymal proteins vimentin and fibronectin[34]. Indeed both proteins were induced as a consequence of Slug expression and consistent with other studies Slug also mediated a repression in beta-catenin, a protein involved in both cell-cell adhesion and *Wnt* signalling[35].

Abrogating Slug induction may thus represent a potential therapeutic strategy since it appears central to the EMT phenotype observed in esophageal adenocarcinoma. A potential approach might be to silence or block known inducers of Slug. In this regard, the extracellular signals *FGF*, *TGF-beta*, and *Wnt*, which have been reported to be over-expressed and implicated in the pathogenesis of esophageal adenocarcinoma, have been found to induce *Slug* expression[13,14,36-38]. Thus abrogating the expression of these extracellular signals might represent a mechanism of repressing *Slug* and potentially mediating a mesenchymal to epithelial transition which is likely to impact on development of disease and ultimately patient survival.

In summary of the EMT regulators examined, *Slug* appears to be the only transcription factor over-expressed in esophageal carcinogenesis. A downstream consequence of *Slug* overexpression is likely to be silencing of *E-cadherin* and ultimately in concert with other signalling molecules induction of invasion and metastasis.

COMMENTS

Background

The incidence of esophageal adenocarcinoma is currently rising faster than any other cancer in the Western world though the cause of this increase is largely unknown. Progression of this disease is associated with a repression in the cell adhesion molecule E-cadherin; a crucial event in invasion and metastasis.

Research frontiers

E-cadherin silencing is a common event in nearly all epithelial malignancies and in several cancers this is either due to mutation, gene deletion or promoter methylation. However, how *E-cadherin* is silenced in esophageal adenocarcinoma has not been unequivocally addressed. In this study, the authors demonstrate that the overexpression of *Slug* could be a potential mechanism for mediating *E-cadherin* repression.

Innovations and breakthroughs

Recent reports have highlighted the importance of epithelial mesenchymal regulators including *Snail*, *Slug* and *Twist* in gastrointestinal carcinogenesis. In particular in esophageal squamous cell cancers, Slug is over-expressed. This is the first study to report that Slug is also over-expressed in esophageal adenocarcinomas. Furthermore, our *in vitro* studies would suggest that this protein may be the cause of the repression in E-cadherin observed in this cancer.

Applications

By understanding how Slug is induced and by blocking its expression, this study may represent a future strategy for therapeutic intervention in the treatment of patients with esophageal adenocarcinoma.

Terminology

Slug, Snail and Twist are all proteins involved in the process called epithelial mesenchymal transition. This is when epithelial cells lose cell-cell adhesion and behave more like fibroblasts. Such a mechanism is thought to be crucial in invasion and metastasis of cancer. Non-surprisingly, the cell adhesion molecule E-cadherin is repressed during this process.

Peer review

The authors examined the expression of E-cadherin and its repressors; Snail, Slug and Twist in squamous esophagus, Barrett's metaplasia and esophageal adenocarcinoma. It revealed that *Slug* was increased in adenocarcinoma and in OE33 cells, the increase of *Slug* expression was inversely correlated to *E-cadherin* expression (mRNA and promoter activity) and induced EMT. The results are interesting and may represent a molecular mechanism of esophageal carcinogenesis.

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Frequent mutations of the CA simple sequence repeat in intron 1 of *EGFR* in mismatch repair-deficient colorectal cancers

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Abstract

AIM: To investigate the polymorphic simple sequence repeat in intron 1 of the epidermal growth factor receptor gene (*EGFR*) (CA-SSR Ⅰ), which is known to affect the efficiency of gene transcription as a putative target of the mismatch repair (MMR) machinery in colorectal tumors.

METHODS: The CA-SSR Ⅰ genotype was analyzed in a total of 86 primary colorectal tumors, selected upon their microsatellite instability (MSI) status [42 with high frequency MSI (MSI-H) and 44 microsatellite stable (MSS)] and their respective normal tissue. The effect of the CA-SSR Ⅰ genotype on the expression of the *EGFR* gene was evaluated in 18 specimens using quantitative real-time reverse transcription PCR and immunohistochemistry.

RESULTS: Mutations in CA-SSR Ⅰ were detected in 86% (36 of 42) of MSI-H colorectal tumors and 0% (0 of 44) of MSS tumors, indicating the *EGFR* gene as a novel putative specific target of the defective MMR system ($P < 0.001$). Impaired expression of *EGFR* was detected in most of the colorectal tumors analyzed [6/12 (50%) at the mRNA level and 15/18 (83%) at the peptide level]. However, no association was apparent between *EGFR* expression and CA-SSR Ⅰ status in tumors or normal tissues.

CONCLUSION: Our results suggest that CA-SSR Ⅰ sequence does not contribute to the regulation of *EGFR* transcription in colon, and should thus not be considered as a promising predictive marker for response to *EGFR* inhibitors in patients with colorectal cancer.

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Key words: Epidermal growth factor receptor; Microsatellite instability; Allelic imbalance; Gene polymorphism; Expression; Colorectal cancer

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INTRODUCTION

The epidermal growth factor receptor EGFR (HER1/ERBB1) is a member of the erbB family. It plays a pivotal role in the control of processes occurring during oncogenesis and tumor progression, such as cell survival, proliferation,

angiogenesis and metastasis (for review[1]). Overexpression of this receptor is observed in a large range of human cancers, correlating with an aggressive tumor phenotype, poor prognosis and decreased survival[2,3]. EGF, which is faintly expressed in normal colon, is seen overexpressed in approximatively 25%-77% of colorectal cancers depending on the technique used[2]. Overexpression of EGFR in colorectal tumors led to the development of targeted therapies against this receptor. Cetuximab (Erbitux®), a monoclonal antibody that specifically blocks the EGFR, has been recently approved for treatment of patients with chimiotherapy-refractory metastatic colorectal cancer. Clinical trials showed response rates of approximately 10% for cetuximab alone and 20% for cetuximab in combination with irinotecan[4]. A recent key finding was a lack of a close correlation between EGFR expression in colorectal tumors, at least as measured by immunohistochemistry, and clinical response[5]. There is therefore urgency to identify robust biological predictive markers for response to *EGFR* inhibitors.

Mechanisms underlying EGFR overexpression in colon are not fully understood. This overexpression can be attributed in part to gene amplification as previously shown in glioblastoma[6]. However, EGFR amplification is infrequent in colorectal cancers[7]. Furthermore, the *EGFR* gene contains a highly polymorphic simple sequence repeat in intron 1 (CA-SSR I), which consists of a variable number of CA dinucleotide repeats[8]. This sequence has been shown to modulate gene transcription in skin, mammary, head and neck, and gastric cell lines, with transcription activity declining with increasing number of CA repeats[9,10]. This finding was confirmed in breast tumors[11]. The mechanism is not fully understood, but it has been proposed that CA-SSR I could act like a joint, bringing the promoter in proximity to a putative repressor protein bound downstream of the repeat sequence[12]. Moreover, a recent study performed on head and neck cell lines reported that cell lines with a lower number of CA repeats are more sensitive to the inhibitory effect of erlotinib (Tarceva®), a small *EGFR* tyrosine kinase inhibitor[10]. Transferring these data to *in vivo*, this would lead to the hypothesis that patients would display different responses to therapy with EGFR inhibitors depending on the status of their tumor for CA-SSR I . Altogether, these data present preliminary evidence that the genotype of CA-SSR I determined in the tumor may be a promising marker to predict the clinical response to anti-EGFR therapy. However, very little is known regarding CA-SSR I in colorectal cancer.

Colorectal cancers can be subdivided into two major categories reflecting different mechanisms of carcinogenesis[13]. Almost 85% of colorectal tumors display chromosomal instability, which is characterized by aneuploidy, chromosomal rearrangements, allelic imbalance (AI) with amplifications and losses of heterozygosity (LOH). On the other hand, 12%-15% of colorectal tumors show high frequency microsatellite instability (MSI-H), which reflects a failure in the DNA mismatch repair (MMR) system. Most of them occur sporadically, but they can also occur (2%-5%) in the context of hereditary non-polyposis colorectal cancer (HNPCC). A great number of microsatellite repeat sequences distributed throughout the genome have been found (by targeted or systematic search) to be affected by MSI. However, only a small number are accepted as real targets of MSI, as they are assumed to play a role in the progression of MMR-deficient tumors[14-17]. Most of them have been described in coding sequences of genes implicated in the regulation of cell growth or cell survival. However, some non-coding repeat sequences affecting gene expression have also been proposed as targets of MMR[18].

In this context, we investigated the CA-SSR in intron 1 of *EGFR* in a total of 86 colorectal cancers selected upon their MSI status (42 MSI-H and 44 MSS) and in their respective normal tissue. Furthermore, we evaluated the influence of CA-SSR I mutations and length polymorphisms on EGFR expression and tested the hypothesis that CA-SSR I could be a target of MSI in MMR-deficient colorectal tumors.

MATERIALS AND METHODS

Patients and tumors

A total of 86 colorectal cancers were included in the study. Cancers were selected among 250 colorectal tumors we previously analyzed for MSI using the Bethesda panel[14] or the pentaplex mononucleotide panel[19] in order to enrich our series into MSI-H tumors. They comprised 42 MSI-H tumors and 44 microsatellite stable (MSS) tumors. All were from patients who had undergone surgery for primary colorectal cancer in various hospitals from northern France. Patients followed the revised Bethesda criteria for MSI testing and identifying individuals with HNPCC (for review, see[20]).

CA-SSR I genotyping and data analysis

CA-SSR I genotype analysis was conducted on tumoral tissue DNA and on respective constitutional DNA from normal colon taken at a distance from the tumor. DNA was extracted from formalin-fixed paraffin-embedded tissues using the EZ1 DNA Tissue kit with the BioRobot® EZ1 (Qiagen, Courtaboeuf, France). CA-SSR I was then amplified by PCR using previously described primers[10], the forward primer being labelled with 6-FAM at the 5' end. Genotypes were resolved on Applied Biosystems 377XL analyzer using GeneScan software (Applied Biosystems, Courtaboeuf, France). The (CA)n dinucleotide repeat length was confirmed by direct sequencing of some samples. Alleles were compared between the tumor and the normal matching tissue. The tumor was considered as presenting MSI for CA-SSR I when additional alleles were observed compared to the matching normal tissue. The tumor was considered as presenting AI when the AI score was < 0.6 or > 1.67, as determined previously by Canzian *et al*[21]. AI score = T1 × N2 / T2 × N1, where T is tumor, N is normal and 1 is the area under the peak corresponding to the shorter allele, 2 is the area under the peak corresponding to the longer allele. This threshold allows for the possibility of contaminating normal tissue or tumor heterogeneity in some samples[21].

Quantitative real-time reverse transcription-PCR (QRT-PCR) analysis

QRT-PCR analysis was conducted in 12 out of the 86 patients for whom quick-frozen tumor and normal colonic tissues were available in the tissue bank of Lille (CHRU de Lille). Total RNA was extracted from frozen tissues with the NucleoSpin® RNA II kit (Macherey-Nagel) and treated with RNase-free DNase to remove any contaminating genomic DNA. RNA (900 ng) was reverse transcripted using oligodT and the Advantage® RT-for-PCR kit (BD Biosciences Clontech) according to the manufacturer's instructions. Expression of EGFR was investigated by QRT-PCR using SybrGreen methodology on a LightCycler (Roche) and previously described primers[22]. β-actin mRNA was used as the endogenous control gene. All real-time assays were performed in triplicate. Relative expression of EGFR in tumor samples (compared with their respective normal tissue) and in normal tissues (compared with normal tissues with a 16/16 genotype) was calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemical (IHC) analysis

Immunohistochemical analysis was performed using the monoclonal anti-EGFR antibody Clone 31G7 from Zymed Laboratories (CliniSciences, Montrouge, France). The staining procedure was conducted using an automated immunostainer Benchmark XT (Ventana medical Systems, Strasbourg, France) and a three-step indirect process based on the biotin-streptavidin-peroxidase method. Briefly, after proteinase pretreatment (Ventana medical Systems), tissue sections were incubated with fresh 3% hydrogen peroxide in methanol to block endogenous peroxidase. The sections were then sequentially incubated with the mouse monoclonal anti-EGFR antibody (dilution 1:20, 20 min), a biotinylated rabbit anti-mouse antibody and streptavidine-peroxidase conjugate. The sections were then developed with diaminobenzidine and counterstained with hematoxylin. A section of esophagus known to express EGFR was used as a positive control.

Specimens were examined for the distribution of the immunostaining (membranous and/or cytoplasmic). Only cells showing membranous staining (alone or in association with cytoplasmic staining) were considered as positive and scored. EGFR expression was assessed according to the percentage of positive cells using the 0 to 3+ scale as follows: score 0, absence of positive cells; 1+, > 0%-1%; 2+, 2%-50%; 3+, > 50%.

Statistical analysis

Ordinal data were compared with the χ^2 test or Fisher's exact test, as appropriate. Continuous data, specifically the relationship between the number of CA repeats in normal tissues and EGFR mRNA expression levels was assessed by the Mann-Whitney *U* test. All statistical tests were two-sided with the threshold of significance set at $P < 0.05$.

RESULTS

CA-SSR I genotypes

Germline alleles determined in normal colonic tissues ranged in length from 14 to 21 CA repeats. Heterozygosity was 58% (50/86) for this series of patients. The most common alleles consisted of 16 CA repeats [85 of 172 chromosomes (49%)], 18 CA [31/172 (18%)], and 20 CA [38/172 (22%)]. The most common genotype was found to be homozygous for 16 CA repeats [24/86 (28%)] (Table 1).

Table 1 Allelic and genotype distribution of *EGFR* CA-SSR I polymorphism in normal tissues of patients with colorectal tumors *n* (%)

Genotypes in normal colon	CA-SSR I status of the tumor[a]		Total (*n* = 86)
	CA-SSR I MSI (*n* = 36)	CA-SSR I MSS (*n* = 50)	
14/20	1 (2.8)	0 (0)	1 (1.2)
15/20	1 (2.8)	0 (0)	1 (1.2)
16/16	10 (27.8)	14 (28)	24 (27.9)
16/17	2 (5.6)	2 (4.0)	4 (4.7)
16/18	4 (11.1)	11 (22.0)	15 (17.4)
16/19	0 (0)	1 (2.0)	1 (1.2)
16/20	6 (16.7)	9 (18)	15 (17.4)
16/21	0 (0)	2 (4.0)	2 (2.3)
17/20	0 (0)	1 (2.0)	1 (1.2)
18/18	2 (5.6)	2 (4.0)	4 (4.7)
18/19	1 (2.8)	0 (0)	1 (1.2)
18/20	2 (5.6)	3 (6.0)	5 (5.8)
18/21	1 (2.8)	1 (2.0)	2 (2.3)
19/21	1 (2.8)	0 (0)	1 (1.2)
20/20	4 (11.1)	3 (6.0)	7 (8.1)
20/21	1 (2.8)	0 (0)	1 (1.2)
21/21	0 (0)	1 (2.0)	1 (1.2)

[a]$P = 0.673$, CA-SSR I MSI *vs* CA-SSR I MSS according to the allele lengths (patients with at least one allele ⩾ 20 CA (17/19) *vs* both alleles < 20 CA (19/23), respectively) (χ^2 test).

Comparison of alleles in MSS colorectal tumors and respective normal colonic tissues showed a perfect correlation of allele lengths. In contrast, instability of CA-SSR I was detected in 36 of the 42 (86%) MSI-H tumors (compared to 0 of the 44 (0%) MSS tumors, $P < 0.001$, χ^2 test) (Table 2). Instability consisted in shortening of the CA-SSR I in 6 tumors (6/36, 17%), elongation of the CA-SSR I in 18 tumors (18/36, 50%), and combined shortening and elongation of the CA-SSR I in 12 tumors (12/36, 33%) (Figure 1). In most cases, tumors displayed multiple alleles in addition to normal germline alleles, hardly suggesting tumor cell heterogeneity. New alleles ranged in length from 12 CA repeats to 27 CA repeats for major alleles (from 12 CA repeats to 29 CA repeats for all alleles). Tumors from patients presenting at least one long allele with ⩾ 20 CA in the germline were not more frequently unstable than tumors from patients with both alleles shorter (< 20 CA) [17/19 (89%] *versus* [19/23 (83%)], ($P = 0.673$, χ^2 test) (Table 1), indicating that long CA-SSR I alleles are not particularly prone to MSI.

Moreover, comparison of alleles in tumors and respective normal tissues allowed detection of AI in 5 of the 30 (17%) MSS tumors from patients heterozygous for CA-SSR I and that were thus informative (AI scores = 0.47, 0.59, 2.27, 3.03, and 4.46, respectively) (Table 2).

Relationship between CA-SSR I genotype and EGFR mRNA levels

To investigate the influence of CA-SSR I mutations and allele lengths on *EGFR* gene transcription, we used QRT-

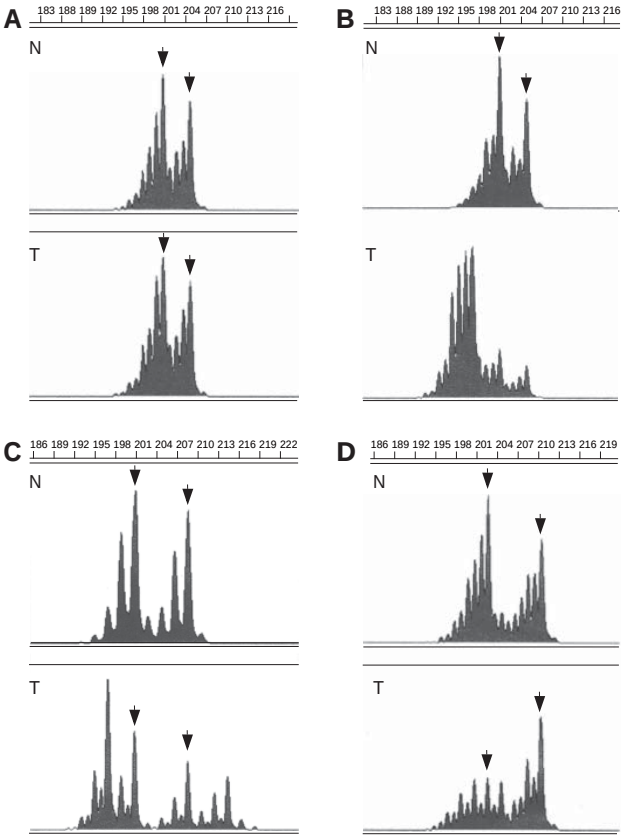


Figure 1 Examples of MSI and AI of EGFR CA-SSR I in colorectal tumors. (**A**, **B**, **C**, and **D**) show heterozygosity for CA-SSR I in normal tissue (N). (**A**) shows no instability nor AI (AI score = 0.99) in colorectal tumor (T) of the same patient; (**B**) shows MSI with additional shortened alleles in colorectal tumor; (**C**) shows MSI with combined shortened and elongated alleles in colorectal tumor; and (**D**) shows AI (AI score = 3.03) in colorectal tumor. Arrows indicate germline alleles.

PCR to evaluate EGFR mRNA expression in both tumor and normal tissues from 12 patients we previously analyzed for CA-SSR I genotype. Comparison between tumors and matching normal tissues showed impaired expression of *EGFR* mRNA in half of the cases (6/12 with fold change > 2 or < 0.5) (Table 3). However, no association was apparent between *EGFR* mRNA expression levels measured in tumors and the length of CA-SSR I alleles, the presence of CA-SSR I instability or AI. Furthermore, EGFR mRNA expression levels measured in normal tissues were similar among individuals, whatever their CA-SSR I genotype status, indicating no association between expression of *EGFR* mRNA and the number of CA repeats in normal colon (two by two comparisons, $P > 0.468$, Mann-Whitney *U* test) (Figure 2).

Relationship between CA-SSR I genotype and EGFR peptide expression by immunohistochemistry

EGFR peptide expression was evaluated by immunohistochemistry in 18 tumors previously analyzed for CA-SSR I genotype. Overexpression of *EGFR* with membranous staining or both membranous and cytoplasmic staining (score ⩾ 2+) was observed in 15 out of the 18 (83%) tumors analyzed (Table 3, Figure 3). *EGFR* immunostaining level appeared to be not associated with the length of CA-SSR I alleles, the presence of CA-SSR I instability or AI. Moreover, no association could be observed between *EGFR* immunostaining and *EGFR* mRNA expression levels. Noteworthy, cytoplasmic staining alone (without membranous staining) was observed in 6 of the 7 tumors (86%) with CA-SSR I instability compared to 0 of the 11 tumors (0%) without CA-SSR I instability ($P < 0.001$, χ^2 test). This observation could not be related to a particular histopathological feature such as the degree of differentiation.

Table 2 Status of EGFR CA-SSR I in patients with MSI-H and MSS colorectal tumors *n/n* (%)

	CA-SSR I MSI[b]	CA-SSR I MSS[b]	CA-SSR I MSS[1]	
			AI+	AI-
MSI-H (n = 42)	36/42 (85.7)	6/42 (14.3)	0/1 (0)	1/1 (100)
MSS (n = 44)	0/44 (0)	44/44 (100)	5/30 (16.7)	25/30 (83.3)

[1]Tumors from informative patients for AI score determination, i.e. heterozygous for CA-SSR I. [b]$P < 0.001$, regarding the comparison of CA-SSR I MSI *vs* CA-SSR I MSS distribution according to the MSI status (χ^2 test).

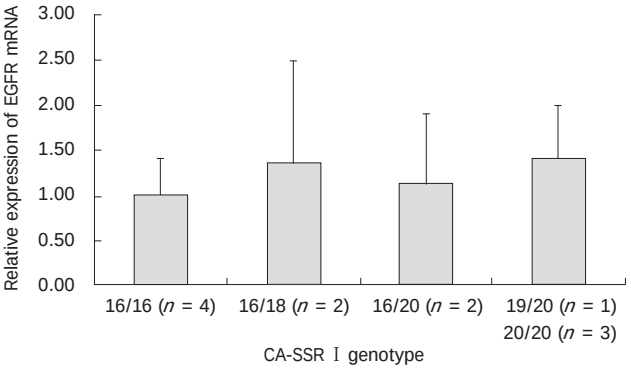


Figure 2 Graphical representation of the influence of the CA-SSR I genotype on EGFR mRNA expression in normal colon. Expression levels were determined by QRT-PCR. Relative expression levels are given in percent, relative to expression levels determined in normal colons with a 16/16 genotype. Median values with the corresponding standard deviations are indicated. Two by two comparisons, $P > 0.468$ (Mann-Whitney *U* test).

DISCUSSION

Here, we have analyzed primary colorectal tumors selected by their MSI status (42 MSI-H and 44 MSS) and their respective normal tissue for CA-SSR I mutations and length polymorphisms. Furthermore, we evaluated the effect of CA-SSR I genotype with respect to EGFR expression in colorectal tumors.

The distribution of CA-SSR I alleles and genotypes that were determined in normal tissues were similar to those reported previously in Caucasian reference pedigrees, with the most common alleles consisting of 16, 18, and 20 CA repeats and the most common genotype being homozygous for 16 CA repeats[8]. Interestingly, whereas allele lengths were perfectly conserved in MSS tumors, frequent instability of CA-SSR I was detected in MSI-H tumors. This instability consisted in shortening, elongation, or combined shortening and elongation of the CA-SSR I;

Table 3 Relationship between *EGFR* CA-SSR I genotypes and EGFR expression levels in colorectal tumors

	CA-SSR genotypes[1]		CA-SSR I status[2] of the tumor	EGFR expression	
	Normal colon	Tumor		QRT-PCR[3]	IHC[4]
1	16/16	16/20	MSI	0.87	++ m
2	16/16	16/16	MSS	0.70	++ m
3	16/18	16/18	MSS	2.68	++ m
4	16/16	14/16/20/(21/22/23)	MSI	5.10	+++ m, c
5	20/20	16/25/26/27	MSI	0.27	++ m, c
6	20/20	20/20	MSS	0.76	++ m
7	16/20	16/20	MSS (AI +)	1.11	+++ m
8	20/20	20/20	MSS	0.48	++ m
9	16/20	16/20	MSS	3.68	++ m
10	16/16	16/16	MSS	1.32	++ m
11	16/18	16/18	MSS (AI +)	0.47	+ m
12	19/21	19/21/(22/23)	MSI	1,57	+++ m, c
13	16/18	14/16/18	MSI	ND	++ m, c
14	14/20	12/14/20/(22/23)	MSI	ND	++ m, c
15	16/18	16/18/(23/24)	MSI	ND	+++ m, c
16	16/20	16/20	MSS	ND	-
17	16/16	16/16	MSS	ND	-
18	18/18	18/18	MSS	ND	++ m

[1]CA-SSR I alleles determined in colorectal tumors and corresponding normal tissues (germline alleles). All observed alleles are indicated, with minor alleles in brackets; [2]MSI, presence of CA-SSR I instability; MSS, absence of CA-SSR I instability; AI +, presence of allelic imbalance; [3]Relative expression of EGFR mRNA levels (tumor relative to matching normal tissues), as assessed by QRT-PCR and calculated using the $2^{-\Delta\Delta Ct}$ formula. Values corresponding to a fold change > 2 or < 0.5 are indicated in bold. ND: not determined; [4]Expression of EGFR peptide determined by immunohistochemistry. Score 0, 0% of positive cells with membranous staining; 1+, > 0%-1%; 2+, 2%-50%; 3+, > 50%. m: Membranous staining; c: Cytoplasmic staining.

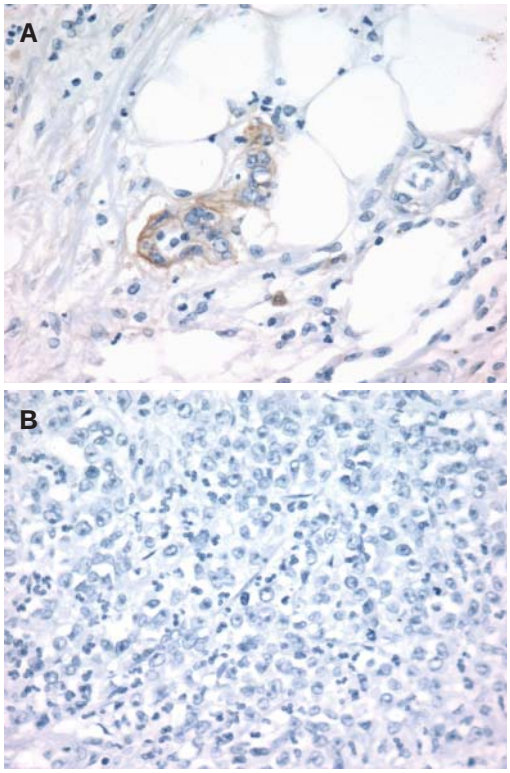


Figure 3 Examples of EGFR immunohistochemical stainings in colorectal tumors harboring identical EGFR CA-SSR I status and genotype. (**A** and **B**) are CA-SSR I MSS colorectal tumors with genotype 16/20; (**A**) shows a moderate membranous staining for EGFR (score ++), whereas (**B**) is negative (x 400; hematoxylin counterstain).

it was independent of the length of alleles determined in normal tissues. The frequency of instability was particularly high, accounting for 86% of MSI-H colorectal tumors. *EGFR* CA-SSR I may thus be of interest as an alternative marker for the determination of the MSI status in colorectal tumors[14]. It is noteworthy that this study was performed on tumors without previous microdissection. Enriching for tumor cells using microdissection would still enhance the likehood of detecting novel shifted alleles. One could argue that this study was performed on tumors of patients following the Bethesda criteria, and thus suspected to have a HNPCC syndrome, introducing some biases in the data. However, previous studies revealed similar frequencies in instability for a given marker in both sporadic and HNPCC tumors[16]. Moreover, 15 of 24 patients with MSI-H who were screened for germline mutations in MMR genes by direct sequencing and by multiplex ligation-dependant probe amplification displayed mutations in *MLH1*, *MSH2* or *MSH6*, indicating that approximately 1/3 of MSI-H tumors tested here were likely to be sporadic (data not shown). Of note, CA-SSR I analysis of two MSI-H keratoacanthomes and one MSI-H endometrial tumor from three independent HNPCC patients showed CA-SSR I instability in one keratoacanthome indicating that CA-SSR I instability is probably not restricted to colorectal tumors (data not shown). To our knowledge, there has been no previous report demonstrating such alteration of *EGFR* CA-SSR I polymorphism in MSI-H tumors.

Most of the non-coding repeat mutations observed in MSI-H tumors are considered as background events, since they are unlikely to participate to oncogenesis processes. In this respect, mutation frequencies in these sequences, in the absence of selection pressure, are generally low[14,15,17]. However, mutations in non-coding sequences may contribute to the manifestation of the malignant phenotype if they occur in gene regulatory regions that control levels of expression[17,23]. For example, a T repeat in intron 4 of the *MRE11* gene is frequently mutated in MSI-H tumors. These mutations have been shown to cause a splicing defect with reduced mRNA and protein levels[18]. Here, we have shown that 86% of MSI-H colorectal tumors harbor mutations of CA-SSR I , which is indicative of a possible positive selection pressure. Moreover, the CA-SSR I sequence has been shown in various tissues and cell lines to modulate *EGFR* gene transcription[9-11]. These findings led us to consider *EGFR* as a novel putative target gene of the defective MMR machinery and to hypothesize that *EGFR* CA-SSR I mutations, as well as CA-SSR I length polymorphism, could alter the transcriptional activity of the *EGFR* gene and explain, at least in part, variations of EGFR expression between colorectal tumors.

In addition to the presence of frequent CA-SSR I mutations in MSI-H tumors, CA-SSR I analysis allowed us to detect AI in 17% of MSS tumors. *EGFR* gene amplification has been recently shown to occur in 12% to

31% of colorectal tumors, as determined by chromogenic[7] and fluorescent *in situ* hybridization[24]. Therefore, the presence of AI in some MSS tumors probably reflects amplification of the *EGFR* gene in these tumors, as demonstrated previously for breast tumors[25]. Interestingly, Moroni *et al*[24] have shown that assessment of *EGFR* copy number might identify patients who are likely to respond to cetuximab.

In order to evaluate potential functional consequences of CA-SSR I mutations and polymorphisms, we investigated EGFR expression at both the mRNA and peptide levels. As expected, EGFR peptide was found to be overexpressed in most of the tumors analyzed (83% in the present series). EGFR mRNA expression was also altered in most colorectal tumors, as assessed by QRT PCR. However, *EGFR* mRNA and peptide expression appeared to be not associated with the CA-SSR I allele length, nor with the presence of MSI or AI. Our data regarding *EGFR* peptide expression are in accordance with those obtained by McKay *et al*[26] who showed no association between the distribution of CA-SSR I alleles in colorectal tumors and *EGFR* peptide expression. More intriguingly, the CA-SSR I genotype identified in normal colon samples was not predictive of *EGFR* mRNA expression levels. Our results differ from those obtained in other tissues. *EGFR* transcription activity has been shown *in vitro* and *in vivo* in mammary cell lines to decline with increasing number of CA repeats[9]. In accordance, *EGFR* peptide expression was subsequently found in breast tumors to correlate with the length of CA-SSR I alleles[11]. A direct correlation between *EGFR* expression levels and CA-SSR I has also been demonstrated in skin, gastric, and head and neck cell lines[9,10], but these findings remain to be confirmed in corresponding tissues. Altogether, our present data hardly suggest that in colorectal cancer and normal colon, unlike in breast cancer, CA-SSR I does not contribute to the regulation of *EGFR* transcription.

In conclusion, we have shown that the CA-SSR polymorphism in intron 1 of *EGFR* is frequently altered in MMR-deficient colorectal tumors, as well as in a subset of MSS tumors However, *EGFR* CA-SSR I polymorphisms and mutations seem to be not associated with EGFR expression. Our data indicate that the *EGFR* gene is more likely to be a 'bystander' rather than a 'real functional' target of the MMR machinery. Given the present data, CA-SSR I should not be considered as a promising marker for predicting the clinical response to EGFR inhibitors in patients with colorectal tumors.

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COMMENTS

Background

Cetuximab (Erbitux®), a monoclonal antibody that specifically blocks the epidermal growth factor receptor (*EGFR*), has been recently approved for treatment of patients with chimiotherapy-refractory metastatic colorectal cancer. Clinical trials showed limited response rates for cetuximab alone or in combination with irinotecan. There is therefore urgency to identify robust biological predictive markers for response to EGFR inhibitors.

Research frontiers

Our article focuses on genetic alterations of the *EGFR* gene in colorectal cancers.

Innovations and breakthrought

The polymorphic simple sequence repeat in intron 1 of the *EGFR* gene (CA-SSR I) has been investigated in various cancers, including colorectal cancers. No abnormality has been found in this sequence, except in breast cancers in which allelic imbalance has been extensively described. The present study shows very frequent mutations of CA-SSR I in mismatch repair-deficient colorectal cancers, indicating the *EGFR* gene as a novel putative specific target of the defective mismatch repair system.

Applications

Impaired expression of EGFR was detected in most of colorectal tumors analyzed, but without apparent association with CA-SSR I status. Although these results are preliminary, they hardly suggest that CA-SSR I sequence does not contribute to the regulation of EGFR transcription in colon and should thus not be considered as a promising predictive marker for response to *EGFR* inhibitors.

Peer review

The manuscript from Dr. Buisine *et al* presents an interesting review of mismatch repair deficient cancers looking at polymorphism and mutations of the CA simple sequence repeat in intron-1 of EGFR receptor.

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COLORECTAL CANCER

Predictive value of Ki67 and p53 in locally advanced rectal cancer: Correlation with thymidylate synthase and histopathological tumor regression after neoadjuvant 5-FU-based chemoradiotherapy

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Abstract

AIM: To investigate the predictive value of Ki67 and p53 and their correlation with thymidylate synthase (TS) gene expression in a rectal cancer patient cohort treated according to a standardized recommended neoadjuvant treatment regimen.

METHODS: Formalin fixed, paraffin embedded pre-therapeutical tumor biopsies (n = 22) and post-therapeutical resection specimens (n = 40) from patients with rectal adenocarcinoma (clinical UICC stage Ⅱ/Ⅲ) receiving standardized neoadjuvant 5-fluorouracil (5-FU) based chemoradiotherapy were studied for Ki67 and p53 expression by immunohistochemistry and correlated with TS mRNA expression by quantitative TaqMan real-time PCR after laser microdissection. The results were compared with histopathological tumor regression according to a standardized semiquantitative score grading system.

RESULTS: Responders (patients with high tumor regression) showed a significantly lower Ki67 expression than non-responders in the pre-therapeutical tumor biopsies (81.2% *vs* 16.7%; $P < 0.05$) as well as in the post-therapeutical resection specimens (75.8% *vs* 14.3%; $P < 0.01$). High TS mRNA expression was significantly correlated with a high Ki67 index and low TS mRNA expression was significantly correlated with a low Ki67 index in the pre-therapeutical tumor biopsies (corr. coef. = 0.46; $P < 0.01$) as well as in the post-therapeutical resection specimens (corr. coef. = 0.40; $P < 0.05$). No significant association was found between p53 and TS mRNA expression or tumor regression.

CONCLUSION: Ki67 has, like TS, predictive value in rectal cancer patients after neoadjuvant 5-FU based chemoradiotherapy. The close correlation between Ki67 and TS indicates that TS is involved in active cell cycle processes.

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Key words: p53; Ki67; Neoadjuvant treatment; Rectal cancer; Thymidylate synthase

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Jakob C, Liersch T, Meyer W, Becker H, Baretton GB, Aust DE. Predictive value of Ki67 and p53 in locally advanced rectal cancer: Correlation with thymidylate synthase and histopathological tumor regression after neoadjuvant 5-FU-based chemoradiotherapy. *World J Gastroenterol* 2008; 14(7): 1060-1066 Available from: URL: http://www.wjgnet.com/1007-9327/14/1060.asp DOI: http://dx.doi.org/10.3748/wjg.14.1060

INTRODUCTION

According to the results of the German Rectal Cancer Study Group (CAO-/AIO-/ARO-94 trial) pre-operative (neoadjuvant) 5-fluorouracil (5-FU) based chemoradiotherapy (CT/RT) is now the recommended therapy regimen for rectal cancer UICC stage Ⅱ/Ⅲ[1].

The antimetabolite 5-FU plays a central role in the treatment of colorectal cancer and, additionally, was shown to enhance the effectiveness of radiation therapy in the treatment of rectal cancer[2,3]. One of the most important molecular targets of 5-FU is thymidylate synthase (TS), which is essential for the de novo DNA synthesis[4].

Several clinical studies have shown that high TS expression is a predictive marker for a low sensitivity to adjuvant 5-FU-based chemotherapy in colorectal cancer.

Furthermore, high TS expression is also a prognostic marker for poor disease free and overall survival in colorectal cancer patients not treated with chemotherapy after surgery[5]. Recently, we demonstrated that high TS gene as well as protein expression predicts low therapy-induced tumor regression in neoadjuvantly treated rectal cancer, thus indicating poor treatment efficacy[6,7]. In addition, we showed that persistent positive lymph node status and high TS gene expression after pre-operative 5-FU-based CT/RT are predictive for the development of cancer recurrence and therefore for an unfavorable prognosis in patients with rectal cancer UICC stage Ⅱ/Ⅲ[8].

Since TS is mandatory for DNA synthesis, there is a close relationship between TS expression and proliferation rate: the faster the cell proliferation, the higher the TS expression and activity[9,10].

The Ki67-antigen recognises the nuclei of proliferating cells throughout the cell cycle, with the exception during the G0 and early G1 phases. Several studies showed that a high S-phase fraction is associated with a greater risk for tumor recurrence and diminished survival in the adjuvant setting, although this has not been universally observed[11,12].

Mutations in p53 were found in 40% to 60% of patients with colorectal cancer[13]. Several clinical studies showed a variable, and particular controversial role of the p53 status with regard to therapy response and prognosis in the neoadjuvant and adjuvant setting, respectively, depending on the kind of treatment and the p53 detection technique used[14-17]. Generally, mutation or overexpression of p53 seems to be associated with an unfavorable prognosis for patientss with locally advanced colon cancer[18-20]. However, there are also investigations with contrary results or without any associations[11,21-24]. Thus, the role of p53 as a predictive or prognostic marker is still controversial.

The aim of this study was to examine the predictive value of p53 and Ki67 alone or in combination with TS in patients with locally advanced rectal cancer neoadjuvantly treated with 5-FU based CT/RT. Furthermore, the association between both markers and TS was investigated.

MATERIALS AND METHODS

Patients and treatment

Surgical specimens from 40 patients (male: $n = 33$; female: $n = 7$; median age = 62 years) with rectal adenocarcinoma (cUICC stage Ⅱ/Ⅲ), histopathologically proven by rectal biopsy between 1998 and 2001 at the Department of General Surgery, University Medical Center, Göttingen, Germany were analyzed[6]. In all patients, locally curative (R0) tumor resection was achieved after standardized pre-operative CT/RT. During radiotherapy with a total dose of 50.4 Gy (single dose 1.8 Gy delivered in 28 fractions) 5-FU was administered as a 120-h continuous intravenous infusion of 1000 mg/m^2 per day during the first and fifth weeks. Standardized surgery including total mesorectal excision was scheduled 5 wk after completion of pre-operative treatment and clinical restaging. All patients of this study received the same therapy protocol according to the CAO-/AIO-/ARO-94 trial of the German Rectal Cancer Study Group[1]. Treatment was completed by post-operative application of 4 cycles of bolus 5-FU chemotherapy. The trial was approved by the medical ethics committee of the University of Göttingen (No 20-9-95).

Assessment of tumor regression

Histopathological tumor regression was graded according to the semiquantitative 5-point tumor regression grading (TRG) system proposed by Dworak *et al*: TRG 0 = no regression; TRG 1 = dominant tumor mass with obvious fibrosis or mucin; TRG 2 = dominantly fibrotic or mucinous changes with few tumor cells or groups; TRG 3 = very few tumor cells in fibrotic or mucinous tissue; TRG 4 = no tumor cells, only fibrotic or mucinous mass (total regression or response)[25]. As previously described, samples with dominant fibrous tissue (TRG 2 to 4) were defined as responders, those with dominant tumor mass (TRG 0 and 1) were defined as non-responders[6].

Microdissection, RNA extraction, and quantitation of TS gene expression

Microdissection of the tumor was performed by laser microbeam technique using the P.A.L.M. Robot-Microbeam (P.A.L.M. Bernried, Germany). Tumors in form of macroclusters (> 3 mm) or tumor areas with little fibrosis were manually microdissected using sterile needles. Extraction of total RNA from formalin-fixed, paraffin-embedded tissue was done as previously described[6]. RNA was transcribed into cDNA using M-MLV reverse trancriptase (Invitrogen, Karlsruhe, Germany) and random hexamers (Amersham Pharmacia, Freiburg, Germany) according to the manufacturer's instructions. TS-cDNA was quantified by TaqMan real-time PCR in relation to beta-actin cDNA using the standard curve method. cDNA obtained from HT29 cell line was used as a standard. Primer sequences were based on the GenBank accession numbers AB004047 (gene sequence of beta-actin) and X02308 (gene sequence of TS). PCR conditions used have been described[26].

Immunohistochemistry

Protein expression was assessed on 2-μm sections of paraffin-embedded tissue samples. Deparaffinization and heat-induced antigen retrieval were done in PT Module™ (PTM) buffer (Lab Vision Corporation, Fremont, CA, USA) in a pressure steam-cooker at 98 ℃ for 45 min. The slides were cooled down at room temperature for 20 min and then loaded onto a Lab Vision Microm 2D automated cell stainer (Microm, Walldorf, Germany). Endogenous peroxidase activity was inhibited by incubating the slides in 3% H_2O_2 for 10 min. The tissues were incubated at room temperature with the primary MIB1 antibody (DAKO, Hamburg, Germany) at a 1:400 dilution for 30 min and the primary p53 D0-1 antibody (Calbiochem, Frankfurt, Germany) at a 1:50 dilution for 30 min, respectively. The slides were then incubated with a biotinylated secondary antibody for 15 min, and after this incubated with avidin-biotinylated peroxidase complex (ABC) for 15 min. The chromogen

Table 1 Tumor regression after neoadjuvant chemoradiotherapy[6]

	Histopathological tumor regression										
	Primary tumor (downsizing)						Tumor stage (downstaging)				
ypT	TRG0	TRG1	TRG2	TRG3	TRG4	ypUICC	TRG0	TRG1	TRG2	TRG3	TRG4
0	-	-	-	-	3	0	-	-	-	-	2
1	-	-	-	1	-	I	-	2	3	4	-
2	-	2	4	6	-	II	1	2	4	3	-
3	1	3	10	8	-	III	-	3	7	7	1
4	-	2	-	-	-	IV	-	-	-	1	-
Total	1	7	14	15	3	Total	1	7	14	15	3

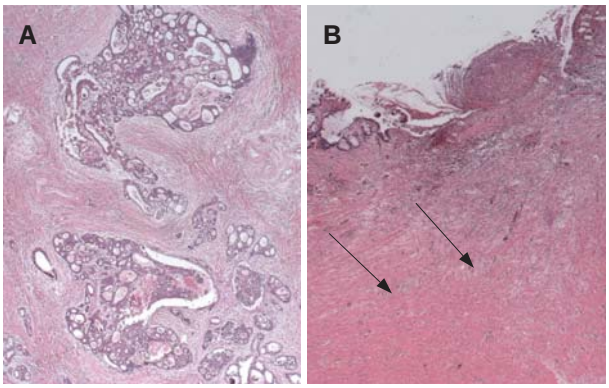


Figure 1 Rectal adenocarcinomas with different tumor regression grade (TRG) after preoperative chemoradiotherapy: dominant tumor with obvious fibrosis in a non-responder, TRG1 (**A**); dominant fibrosis with very few tumor cell groups in a responder, TRG3 (**B**).

3, 3-diaminobenzidine (DAB; 0.6 mg/mL) was applied for 2 min × 4 min. The tissues were counter-stained with Mayer's hematoxylin. In each experiment positive and negative controls were included; isotype controls were done and proved negative.

Evaluation of immunohistochemistry

The immunostained sections were independently reviewed by two investigators (CJ and DEA). Conflicts in scores were resolved by consensus. For the tissue evaluation of Ki-67, each slide was scored based on the percentage of positively stained malignant nuclei. The following ranges were used: 0% to 20%, > 20% to 40%, > 40% to 60%, > 60% to 80%, and > 80% to 100%. According to the recommended classification in previous studies[21,27], samples with Ki67 nuclear staining equal or above 40% were considered having a high proliferative index, whereas nuclear positivity below 40% was considered a low proliferative index. P53 was considered overexpressed when ⩾ 10% of the malignant nuclei were positive. If fewer than 10% of the nuclei were stained, the slide was scored as having normal p53 expression.

Statistical analysis

The correlation between expression levels in tumor biopsies and resection specimens was evaluated with linear regression analysis. The correlation between TS and Ki67 or p53, respectively, was evaluated with the students-*t*-test. For TS gene expression, the maximal χ^2 method was adapted to determine which cut-off value best separates tumors into low and high expression subgroups[28,29]. The correlation between TS, Ki67, and p53 expression and tumor regression was assessed with the chi-square test. The combination of the marker expressions was tested regarding a better discrimination between responders and non-responders with a multivariate discriminant analysis. *P* values < 0.05 were considered statistically significant.

RESULTS

Pre- and post-therapeutical tumor stage and histopathological tumor regression

The results of the histopathological tumor regression after neoadjuvant CT/RT with respect to downsizing and downstaging have been reported previously[6]. The data are summarized in Table 1. According to our criteria, eight tumors were non-responders and 32 were responders. Representative examples of tumors with different regression grades are shown in Figure 1.

TS gene, p53 and Ki67 protein expression

The median of TS gene expression in pre-therapeutical tumor biopsies and post-therapeutical resection specimens was 1.07 and 0.70, respectively.

Tumor cells showed a nuclear staining pattern for Ki67 or p53. Cytoplasmic (without nuclear) immunoreactivity was not encountered. Epithelial cells located in the generative zone and the basal layers of normal rectal mucosa were consistently immunoreactive for Ki67. No apparent p53 expression was detected in the non-neoplastic cells. Representative immunohistochemical staining examples are shown in Figures 2 and 3.

Correlation between TS, Ki67, and p53 expression and tumor regression in pre-therapeutical rectal biopsies and post-therapeutical resection specimens

Low TS gene expression and low Ki67 expression, respectively, were significantly associated with high tumor regression (response) in the pre-therapeutical tumor biopsies as well as in the post-therapeutical resection specimens. No significant association was found between TS gene and p53 protein expression. The results are summarized in Tables 2 and 3.

Comparison of expression levels between pre-therapeutical biopsies and post-therapeutical resection specimens

A significant correlation between pre-therapeutical tumor

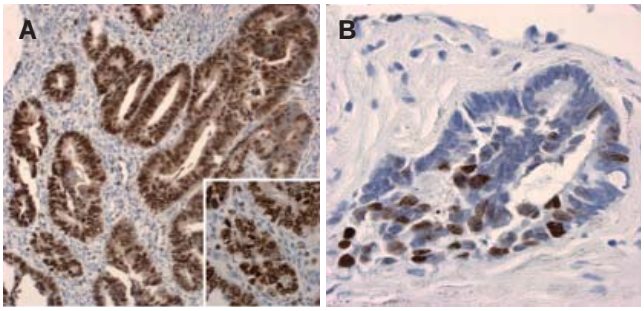


Figure 2 Ki67 expression in post-therapeutical resection specimens. High proliferative index with more than 90% positive nuclei (magnification × 10; insert: × 40) (**A**), and low proliferative index with less than 40% positive nuclei (magnification × 20) (**B**).

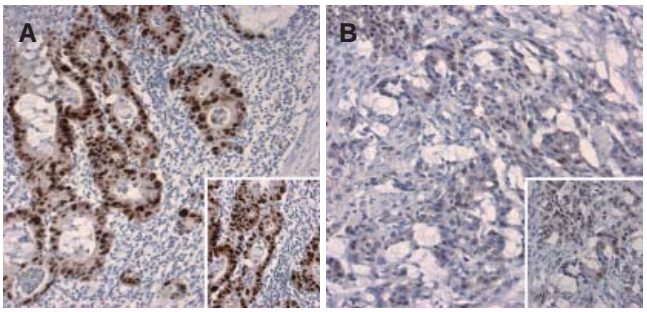


Figure 3 P53 expression in post-therapeutical resection specimens. Overexpression of p53 with more than 75% (**A**) and about 25% positive nuclei (**B**) (magnification x 10; inserts x 20).

biopsies and post-therapeutical resection specimens was observed for the TS gene expression ($P < 0.05$) and p53 expression ($P < 0.05$). No significant correlation was found for Ki67.

Correlation between TS gene expression and Ki67 or p53 expression

High TS mRNA expression was significantly correlated with a high Ki67 index and low TS mRNA expression was significantly correlated with a low Ki67 index in the pre-therapeutical tumor biopsies ($P < 0.01$) as well as in the post-therapeutical resection specimens ($P < 0.05$). No significant correlation was found between TS and p53.

Multivariate analysis

Although both, TS gene and Ki67 expression were significantly correlated with histopathological tumor regression, a combination of the markers did not significantly improve the discrimination between responders and non-responders.

DISCUSSION

There is very little and conflicting data about the predictive value of Ki67 and p53 in rectal cancer after neoadjuvant CT/RT[14-17] and almost no data on the correlation with TS expression. The aim of this study therefore was, to define the predictive value of Ki67 and p53 and their correlation with TS gene expression in a patient cohort treated according to the standardized recommended neoadjuvant treatment regimen[1]. We used histopathologically assessed tumor regression as a surrogate endpoint for early determination of the treatment efficacy[6].

Table 2 Correlations in pre-therapy tumor biopsies

Marker expression	Responders	Non-responders	*P*
TS < 1.20[1]	10	1	
TS ⩾ 1.20	0	3	< 0.05
Ki67 < 40%	13	1	
Ki67 ⩾ 40%	3	5	< 0.05
p53 < 10%	6	3	
p53 ⩾ 10%	10	3	0.60

[1]cut-off value.

Table 3 Correlations in post-therapy resection specimens

Marker expression	Responders	Non-responders	*P*
TS < 0.88[1]	26	2	< 0.01
TS ⩾ 0.88	4	4	
Ki67 < 40%	22	1	< 0.01
Ki67 ⩾ 40%	7	6	
p53 < 10%	10	3	0.98
p53 ⩾ 10%	19	4	

[1]cut-off value.

TS is mandatory for DNA replication, and thus a critical target for fluoropyrimidines. Furthermore, TS is a cell cycle enzyme and is present in proliferating cells[9,10]. Previous observations in cell lines showed that the TS expression and activity in asynchronously growing cancer cells were significantly related to the cell doubling time: the faster the cell proliferation, the greater the expression and activity of TS. The expression and activity of TS were strongly related to the doubling time, and not to the number of cycling cells or to the number of cells in S phase[10]. Rapidly proliferating cells have to synthesise a greater amount of DNA- and consequently need a greater amount of TS- than slowly proliferating cells. Thus, TS expression is closely correlated with the cell proliferation rate. The positive association between TS and Ki67 expression in our study supports this thesis. Additionally, on the basis of our results, this relationship seems not to be influenced by (neoadjuvant) therapy because we found it in the pre-therapeutical biopsies as well as in the post-therapeutical resection specimens. As a consequence of the close relationship of Ki67 and TS, we could show that non-responders had a significantly higher Ki67 expression than responders. The combination of both markers (Ki67 and TS expression) did not significantly improve the response prediction.

In the light of the known relationship between tumor cell kinetic and efficacy of chemotherapy our results are somewhat surprising, since one would expect that rapidly growing tumors with high TS and/or high Ki67 levels would benefit more from chemotherapy. Though Ki67 reflects active cell cycling, it does not necessarily quantitate the speed of the cell cycling process that may be the critical factor in sensitivity to chemotherapy[30]. Thus, it is

conceivable that cancers with relatively high Ki67 levels may still have relatively slow doubling times.

In contrast to the expectation that tumors with high proliferation and therefore high TS expression would benefit most from chemotherapy, high TS expression was associated with poor response to 5-FU and poor survival in most patients with colorectal cancer in the majority of the published studies[5]. Therefore, other-obviously TS-independent - events seem to be involved in 5-FU resistance. Inherent or acquired resistance to 5-FU based chemotherapy is obviously multifactorial, since 5-FU sensitivity is for instance influenced by the expression of dihydropyrimidine dehydrogenase[31], the genetic status of p53[32,33], nuclear factor (NF)-kappa B[34], DNA mismatch-repair genes[35], and cell cycle disturbance[36].

The relationship between TS and p53 was investigated in several clinical studies, mostly in the adjuvant setting. Generally, it has been suggested that mutation of the p53 gene or p53 overexpression (as a surrogate marker for p53 mutation) is significantly and independently associated with resistance to 5-FU therapy and with poor prognosis in colorectal cancer[18-20]. Some studies showed that p53 expression may be used to improve the predictive value of TS for response to 5-FU therapy[21,23,37]. However, some investigations failed to demonstrate such an association[38], and others have shown an improved clinical outcome and overexpression of p53[39].

There are only very rare data of p53 and TS in the neoadjuvant setting. Kamoshida *et al*. demonstrated in gastric cancer neoadjuvantly treated with S-1/cisplatin chemotherapy that high expression of TS and/or p53 in the pre-treatment biopsies predicted chemoresistance[40]. Other investigators[41] were not able to find any correlation between p53 and either TS or tumor regression. Our data do not suggest a predictive value of p53 immunohistochemistry in the neoadjuvant therapy of rectal cancer, either. Like us, most investigators have used immunohistochemistry to detect mutant p53, with the assumption that overexpression of p53 is often associated with a mutation, while the lack of expression is usually associated with a wild-type p53 genotype. This assumption has been validated in approximately 60% to 80% of the cases in which mutational analysis and p53 detection by immunohistochemistry were compared[42,43]. But there may be discrepancies between p53 protein expression and p53 mutation status which may at least partly explain the missing correlation between p53, TS and tumor regression.

In conclusion, we demonstrated that Ki67 has, like TS, predictive value in rectal cancer patients after neoadjuvant 5-FU based CT/RT as responders had a significantly lower Ki67 expression than non-responders. The positive association between Ki67 and TS indicates that TS is involved in active cell cycle processes.

COMMENTS

Background

In locally advanced rectal cancer, neoadjuvant 5-fluorouracil (5-FU) based chemoradiotherapy is now the recommended therapy regimen. One of the most important molecular targets of 5-FU is thymidylate synthase (TS), essential for de novo DNA synthesis. A number of clinical studies have shown that TS expression is a predictive marker in colorectal cancer. Since TS is mandatory for DNA synthesis there seems to be a close relationship between TS expression and other cell cycle related markers like p53 and Ki67, which could serve as predictive markers for response to neoadjuvant 5-FU based therapy in rectal cancer.

Research frontiers

Despite the advances in cancer therapy, there are still a number of patients who do not benefit from treatment. Hence, there is a need to identify novel molecular markers discriminating between patients who will benefit from treatment and those who will not.

Innovations and breakthroughs

To date, there is very little and conflicting data about the predictive value of Ki67 and p53 in rectal cancer after neoadjuvant CT/RT. The results of our study suggest that Ki67, like TS, may be used as a predictive marker in rectal cancer patients treated with neoadjuvant 5-FU based chemoradiotherapy.

Applications

The integration of predictive markers like TS and Ki67 into the therapeutical planning will support the advance of individualized treatment, maximizing therapeutic effect and minimizing exposure toxicity.

Terminology

Prognostic markers distinguish between patients with more or less favourable outcome. Predictive markers determine the likelihood of response to therapy and also the potential toxicity; they are always treatment specific.

Peer review

This paper is valuable particularly to colorectal surgeons, oncologists and gastroenterologists interested in rectal cancer biology and biomarkers. It is overall well designed, well written, with interesting results.

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BASIC RESEARCH

Infant intestinal *Enterococcus faecalis* down-regulates inflammatory responses in human intestinal cell lines

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Abstract

AIM: To investigate the ability of Lactic acid bacteria (LAB) to modulate inflammatory reaction in human intestinal cell lines (Caco-2, HT-29 and HCT116). Different strains of LAB isolated from new born infants and fermented milk, together with the strains obtained from culture collections were tested.

METHODS: LABs were treated with human intestinal cell lines. ELISA was used to detect IL-8 and TGF-β protein secretion. Cytokines and Toll like receptors (TLRs) gene expression were assessed using RT-PCR. Conditional medium, sonicated bacteria and UV killed bacteria were used to find the effecter molecules on the bacteria. Carbohydrate oxidation and protein digestion were applied to figure out the molecules' residues. Adhesion assays were further carried out.

RESULTS: It was found that *Enterococcus faecalis* is the main immune modulator among the LABs by downregulation of IL-8 secretion and upregulation of TGF-β. Strikingly, the effect was only observed in four strains of *E. faecalis* out of the 27 isolated and tested. This implies strain dependent immunomodulation in the host. In addition, *E. faecalis* may regulate inflammatory responses through TLR3, TLR4, TLR9 and TRAF6. Carbohydrates on the bacterial cell surface are involved in both its adhesion to intestinal cells and regulation of inflammatory responses in the host.

CONCLUSION: These data provide a case for the modulation of intestinal mucosal immunity in which specific strains of *E. faecalis* have uniquely evolved to maintain colonic homeostasis and regulate inflammatory responses.

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Key words: Lactic acid bacteria; *E. faecalis*; Inflammation; IL-8; TLRs

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INTRODUCTION

The role of gastrointestinal microflora, such as several strains of lactic acid bacteria (LAB) in priming the immune system during ontogeny to limit allergy and chronic inflammatory responses has been brought to attention in recent years[1,2]. Clinical and experimental studies have put forward that gastrointestinal microflora is essential for maintenance of intestinal homeostasis, and their influence may extend beyond the gut, modifying systemic immunity. Studies using intestinal epithelial cell (IEC) lines have suggested that commensal bacteria may "program" IECs to prevent or down-modulate pro-inflammatory responses by interfering with Toll like receptors (TLRs) expression[3,4]. At least 13 TLRs have been identified in humans. Among all the 13 members of TLRs, TLR3, TLR5, TLR9 are mainly expressed by epithelial cells. They could be activated by pathogens leading to the initiation of inflammation by secreting pro-inflammatory cytokines such as Interleukin-8 (IL-8) and Tumor necrosis factor (TNF)-α[5].

The population of microbes harbored in the human intestine is dynamic and varies with age. It was reported that infants who developed allergy were often found to possess less enterococci in the intestines than healthy infants during the first month of life[6-8]. *Enterococcus faecalis* is a major commensal gastrointestinal flora in humans[9,10]. So far, it is not clear if the depletion of enterococci in allergic children is a cause or effect of allergy. Although there are some reports that *E. faecalis* caused infections in immune deficient mice[11], many studies also showed that *E. faecalis* could suppress the proliferation of intestinal pathogens and thus may prevent infection and the induction of inflammation[12-15]. Another study showed that *E. faecalis* lysate could relieve the clinical symptoms of Japanese cedar pollinosis[16]. However, the

role and effect of *E. faecalis* in inflammation reactions are not clear. It is the intention of this study to shed light on the role and mechanisms of anti-inflammatory effect of enterococci. This knowledge could provide a therapeutic window and development of treatments or prophylaxis of intestinal infectious, inflammatory and allergic reactions and perhaps carcinogenesis.

In the present study, the anti-inflammatory effect of human intestinal LABs isolated from infants and probiotic bacteria with alleged anti-allergic properties were investigated in human intestinal cell lines (Caco-2, HT-29 and HCT116). It is demonstrated that *E. faecalis* is the main immune modulator among the intestinal LABs. However, the effect is limited to only a few strains of *E. faecalis*. Carbohydrate components on bacterial cell walls were the regulators of the inflammatory responses in the host.

MATERIALS AND METHODS

Cell lines, media, and bacterial strains

Caco-2, HT-29 and HCT116 cells were obtained from American Type Culture Collection (Manassas, VA). Caco-2 and HT-29 are human colonic adenocarcinoma cells. Differentiated Caco-2 are enterocyte-like cells[17] whereas HCT116 are human colon carcinoma cells. All cells were cultured at 37℃ in a 5% CO_2 atmosphere. Caco-2 cells were cultured in MEM (GIBCO, Grand Island, NY) containing 20% fetal bovine serum (Bioclot, Germany) and 1% nonessential amino acids (GIBCO). HT-29 and HCT116 cells were cultured in DMEM (GIBCO) containing 10% fetal bovine serum (Bioclot, Germany). All culture media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO).

The origin of the bacterial strains used in the experiment is indicated in Table 1. Lactobacilli were stored in De Man, ROGOSA and SHARPE (MRS) agar (Merck, Darmstadt, Germany) plus 25% glycerol, at -80℃ and grown in MRS broth overnight at 37℃ without shaking. Enterococci were stored in Tryptone Soy broth (TSB) (Oxoid, England) plus 25% glycerol at -80℃ and grown in TSB overnight at 37℃ without shaking. *Streptococcus thermophilus* NCIMB 10387 (10387) was grown on M17 medium (Oxoid, England). *Salmonella typhimurium* 14028 (Salm) was maintained on Luria-Bertani (LB) Agar (BBL, Cockeyville, MD, USA). The freshly cultured bacterial strains were further sub-cultured in a 250 mL conical flask with shaking at 120 r/min overnight. The bacteria were then pelleted by centrifugation, washed using serum-free medium three times and then resuspended in complete medium for use.

Infection of intestinal cells

To obtain a polarized monolayer of intestinal epithelial cells (IECs), 1×10^5 Caco-2 cells were grown in a sterile 24-well plate (Nalge Nunc International, USA) to confluence and then for 2 additional weeks to reach full differentiation and polarization. The culture medium was changed every two days. For HT-29 and HCT116 cell lines, 1×10^5 cells were cultured in sterile 24-well flat-bottom plates (Nalge Nunc International, USA) for 24 h before the infection. For the infection step, Caco-2, HT-29 and HCT116 cells were incubated in fresh medium with bacteria at a multiplicity of infection (MOI) of 100 or 1000 for 6 h. Biological triplicates were done.

RNA extraction and expression analysis by reverse transcription PCR (RT-PCR)

After the infection step, Caco-2, HT-29 and HCT116 cells were washed with cold PBS twice. RNAs were then extracted with a RNA extraction kit (Roche Applied Science, Germany) according to the manufacturer's instruction. Samples were stored at -80℃ before assay. The integrity and quality of RNA were determined by running formaldehyde gel. cDNA were synthesized from a template of 1 μg total RNA using the Superscript Ⅲ (Invitrogen Life Technologies, UK) according to the manufacturer's instruction.

PCR were performed using 2 μL of the cDNA as a template in a final volume of 50 μL. The standard program used was as follows: denaturation for 2 min at 94℃, 35 cycles of 30 s at 94℃, 1 min at 55℃ or 60℃, and 1 min at 72℃, followed by a final elongation for 7 min at 72℃. 0.2 unit of Taq (Invitrogen Life Technologies, UK) was used for one PCR. H_2O was used as a negative control and plasmids containing the specific genes were used as a positive control in the PCR step. After electrophoresis in a 1.2% agarose gel containing Ethidium Bromide, PCR products were quantified using the Gene Tool software (Syngene, UK). The primers used and annealing temperatures are described in Table 2.

In this study, the expression of TLR1, TLR2, TLR3, TLR4, TLR5, TLR9, Tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) and Toll inhibitory protein (Tollip) were measured using RT-PCR. Cytokine gene expression of IL-8, IL-4, IL-10, TGF-β and TNF-α were also detected using this method.

Determination of effecter molecules

For the tests in this part, we chose only four strains of LAB [*E. faecalis* (EC1, EC3, EC15, EC16)] to infect HCT116 cells with a MOI of 1000. The cells were co-cultured with bacteria at 37℃ with 5% CO_2 for 6 h. The supernatants were then harvested for cytokines assay. Unless indicated otherwise all enzymes and chemicals were from Sigma, USA.

Cell wall component and crude cell extract preparation: Before sonication, a protease inhibitior (2 mmol/L phenylmethylsulfonylfluoride (PMSF), 1 mmol/L dithiothreitol (DTT) and 1 mmol/L sodium metavanadate) was added to bacterial suspensions. The bacterial cells were then disrupted by sonication (MSE Soniprep 150, SANYO, Japan) on ice for 10 cycles with 30 s pulses and 1 min rest[18]. Cell debris was pelleted down by centrifugation at 15000 × *g* for 60 min at 0℃. The bacterial culture supernatant was aseptically kept on ice for use. The remaining pellet was resuspended in an appropriate volume of complete DMEM. One mL of each supernatant and 1 mL of each cell debris suspension were added to the cell culture. Biologically triplicates were done on this experiment.

Table 1 The LAB tested for their anti-inflammatory properties

Lactic acid bacteria (LAB)	Origin	Remark
Bifidobacterium adolescentis ATCC15703	Infant intestine	-
B. brevi ATCC15700	Infant intestine	-
B. dentium scardori & crociani ATCC27534	Dental carries	-
B. longum ATCC15707	Adult intestine	-
Enterococcus faecalis EC1	Infant feces Age: 1 mo	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Enterococcus faecalis EC3	Infant feces Age: 3 d	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Enterococcus faecalis EC15	Infant feces Age: 3 d	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Enterococcus faecalis EC16	Infant feces Age: 3 d	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Other 23 strains of enterococci and lactobacilli	Infant feces Age: 3 d to 1 mo	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Lactobacillus acidophilus ATCC4356	Human	-
L. brevis K7	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. brevis T1	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. brevis T6	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. casei ATCC11578	Oral	-
L. casei IS7257	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. casei subsp. *casei* NCIMB11970	Cheese	-
L. casei Shirota	Human	Commercial probiotic
L. delbrueckii subsp. bulgaricus NCIMB11778	Bulgarian yogurt	Commercial probiotic
L. delbrueckii bulgaricus D1	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. lactis subsp. *lactis* B4	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. lactis subsp. *lactis* B9	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. lactis subsp. *lactis* B12	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. lactis subsp. *lactis* K5	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. lactis subsp. *lactis* K6	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. lactis subsp. *lactis* K7	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. paracasei LP33	Fermented milk	Commercial probiotic
Lactobacillus paracasei Chamyto	Yogurt	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. paracasei subsp. *paracasei* ATCC11974	Dental carries	-
L. paracasei subsp. *paracasei* NCIMB8001	-	-
L. rhamnusus NCIMB6375	Oral	
L. rhamnusus GG	Human feces	Commercial probiotic
L. rhamnosus NCIMB 8690	-	-
Leuconostoc mesentroides K13	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Streptococcus thermophilus NCIMB10387	Yogurt	Commercial probiotic

Table 2 PCR Primers and the annealing temperatures used for PCRs

Gene name	Forward	Reverse	Annealing temp (℃)	Length (bp)
β-actin	GGCGACGAGGCCCAGAGCAAGAGAGGCAT	CGATTTCCCGCTCGGCCGTGGTGGTGAAGC	55	460
IL-4	AACACAACTGAGAAGGAAACCTTC	GCTCGAACACTTTGAATATTTCTC	55	276
IL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCTC	55	289
IL-10	ATGCCCCAAGCTGAGAACCAAGACCCA	TCTCAAGGGGCTGGGTCAGCTATCCCA	55	352
TGF-β	TGACAGCAGGGATAACACACT	GTAGGGGCAGGGCCCGAGGCA	55	288
TLR1	CTATACACCAAGTTGTCAGC	GTCTCCAACTCAGTAAGGTG	55	219
TLR2	GCCAAAGTCTTGATTGATTGG	TTGAAGTTCTCCAGCTCCTG	55	346
TLR3	GATCTGTCTCATAATGGCTTG	GACAGATTCCGAATGCTTGTG	55	304
TLR4	CTTTATCCAACCAGGTGC	GGAATGCTGGAAATCCAG	50	650
TLR5	TAGCTCCTAATCTGATG	CCATGTGAAGTCTTTGCTGC	50	437
TLR9	GTCCCCACTTCTCCATG	GGCACAGTCATGATGTTGTTG	55	259
TNF-α	CAGAGGGAAGAGTTCCCCAG	CCTTGGTCTGGTAGGAGACG	55	324
Tollip	CGCGGTACCGCCACCATGGCGACCACCGTCAGC	GCCGGGCCCTGGCTCCTCCCCCATCTG	60	850
TRAF6	GTCGGTACCGCCACCATGAGTCTGCTAAACTGTGAA	CGCGGGCCCCTATACCCCTGCATCAGTAC	60	1600

Conditional medium and cell inserts treatment: In order to test for the location of the active components, the cell culture supernatants obtained from "infection of intestinal cells" were harvested sterilely and were then added to the cell culture prepared as in section "infection of intestinal cells". Supernatants were harvested for further cytokine assay testing. In addition, bacteria themselves were also cultured with the control supernatants obtained from the section "infection of intestinal cells". All the treated cells were then incubated at 37℃ with 5% CO_2 for 6 h. The supernatants were then harvested for cytokines assay.

For the cell insert test, HCT116 cells were plated

in wells of Transwell® (USA) multiple well plate and incubated at 37℃ with 5% CO_2 for 24 h. Accompanying inserts were added 1 h before co-culturing. 100 μL of bacterial suspensions with correct concentration were added to each insert.

Triplicates of each condition were done and supernatants were harvested for the cytokine assay test.

Carbohydrate oxidation and protein digestion of bacterial cell wall test: 4 mL of bacterial cells were pelleted by centrifugation at 15000 g at 0℃ for 10 min. The bacterial cells were washed once in PBS and then suspended in 1.5 mL of one of the following solutions: Solution 1 was 0.05 mol/L sodium m-periodate in buffer A (5 mol/L LiCl in water; 0.1 mol/L citrate-phosphate-0.1 mol/L NaCl) with a final pH 4.5. Solution 2 was 0.05 mol/L sodium iodate in buffer A (pH 4.5). Solution 3 was 5 mg/mL in buffer B (0.05 M Tris-HCl; 0.1 M NaCl) with a final pH 8.0. Solution 4 was 5 mg/mL chymotrypsin in buffer B (pH 8.0).

The bacterial cells were incubated with each of the four solutions (numbered from 1 to 4) for 1 h at 37℃. After that, the bacteria were centrifuged at 15000 g at 0℃ for 10 min, washed twice in 1 mL of PBS and resuspended in 4 mL of complete DMEM. 1 mL of each re-suspension was added to one well containing cell culture. Biological triplicates of each condition were done.

Blocking of specific carbohydrate ligands on cell wall: 3 mL of bacterial cells and broth culture supernatant were separated by centrifugation at 15000 g at 0℃ for 10 min. The bacterial cells were then washed once in PBS and then suspended in 3 mL of one of the following solutions: 15 μg/mL Concanavalin A (ConA) in PBS, 15 μg/mL succinyl Concanavalin A (succinyl-ConA) in PBS and 15 μg/mL *Tetragonolobus purpurea* in PBS. The cells were incubated for 30 min at 37℃, then centrifuged at 15000 *g* at 0℃ for 10 min, washed twice in 1.0 mL of PBS, and resuspended in 3.0 mL of complete DMEM. Treatments were done as described in the section "infection of intestinal cells".

UV killed bacteria: To kill the bacteria, 3 mL of bacterial suspensions were exposed to UV light for 5 min[19] to make sure at least 99% of the bacteria were killed. The killed bacteria were plated on their respective agar plates to ensure cell death. After killing the bacteria, treatments were performed as described in the section "infection of intestinal cells".

Adhesion assay

Monolayers of HCT116 were prepared by inoculating 2-chamber slides (Lab-Tek Chamber Slide, Nunc, Inc., USA) with 1.4×10^5 cells per chamber in 2 mL of complete DMEM 24 h before the adhesion assay. After 24 h, the monolayers were washed twice in 1 mL of sterile PBS. Bacterial strains were prepared and 2 mL of each resuspension was added to a chamber of the slide and incubated at 37℃ with 5% CO_2 for 1 h. After incubation, the monolayers were washed twice with sterile PBS then Gram stained and examined microscopically. The numbers of adherent bacteria per 100 HCT116 cells were counted in 10 random microscope areas.

Cytokine assay

Cytokines in cell culture supernatants were assayed by Enzyme Linked Immunosorbent Assays (ELISAs). After the infection (described in the sections "infection of intestinal cells" and "determination of effecter molecules"), supernatants were harvested and centrifuged for 10 min at 12000 *g* to remove the bacteria. IL-8, IL-4 and IL-10 measurements were performed as the instructions in the ELISAs kits (BD bioscience, San Diego, CA). Individual samples were tested in triplicate and the concentration of cytokines was determined using the standard provided by the manufacture.

For TGF-β assays, 100 μL supernatants were transiently acidified with 2 μL 1 M HCL for 1 h at 4℃, and then neutralized with 2 μL 1 mol/L NaOH. The supernatants were then applied to TGF-β ELISAs (BD bioscience, San Diego, CA) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as means ± SD. Significance of differences was determined using the unpaired Student's *T*-test and analysis of variance. P values < 0.05 were considered to be statistically significant.

RESULTS

A total of 27 strains of LAB were isolated from feces of new born healthy infants aged from 3 d and 1 mo on MRS agar or Slanetz/Bartley media. 29 strains of other LAB were obtained from our collection for the comparison of their modulation of inflammation cytokine (Table 1).

Cytokine secretion in intestinal cell lines after exposure to LAB

To assess the effects of LAB on IL-8 secretion in intestinal cell lines, Caco-2, HT-29 and HCT116 cells were incubated with the respective LAB strains listed in Table 1. The experiments were performed 2-5 times in replicates (Figure 1).

In our study, Caco-2, HT-29 and HCT116 continuously expressed IL-8 at a detectable level. Most of the LAB tested (including strains of *E. faecalis*) showed no significant regulation of IL-8 secretion (data not shown) in these three IECs. *Bifidobacterium*, which was reported to down-regulate TNF-α and IL-8 productions and inhibit NF-κB activation in inflamed mucosa of active ulcerative colitis[20], showed no effect on IL-8 secretion in all the three IECs tested. However, *E. faecalis* (EC1, EC3, EC15, EC16), *L. delbrueckii bulgaricus* D1 (L.bul), *L. rhamnosus* GG (L.GG), *L. paracasei* LP33 (LP33), *L. brevis* T6 (T6) and *Streptococcus thermophilus* NCIMB10387 (10387) downregulated IL-8 secretion in Caco-2 cells (Figure 1). In HT-29 cells, only *E. faecalis* (EC1, EC3, EC15 and EC16) could downregulate IL-8 secretion. Whereas in HCT116, *E. faecalis* (EC1, EC3, EC15, EC16), T6 and LP33 suppressed IL-8 secretion. Strikingly, among the 56 LAB that we have tested, *E. faecalis* (EC1, EC3, EC15 and EC16), which were isolated from new born healthy infants, showed

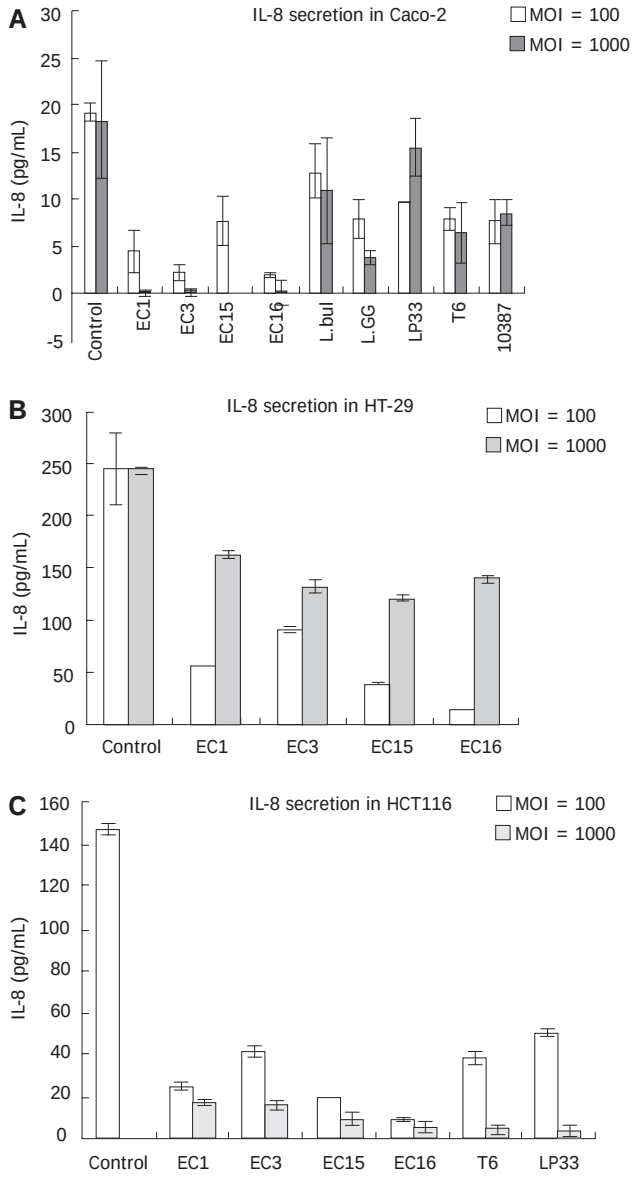


Figure 1 IL-8 production in Caco-2 (**A**), HT-29 (**B**) and HCT116 (**C**) cells treated with LAB for 6 h. Results are shown as mean ± SD of three independent experiments.

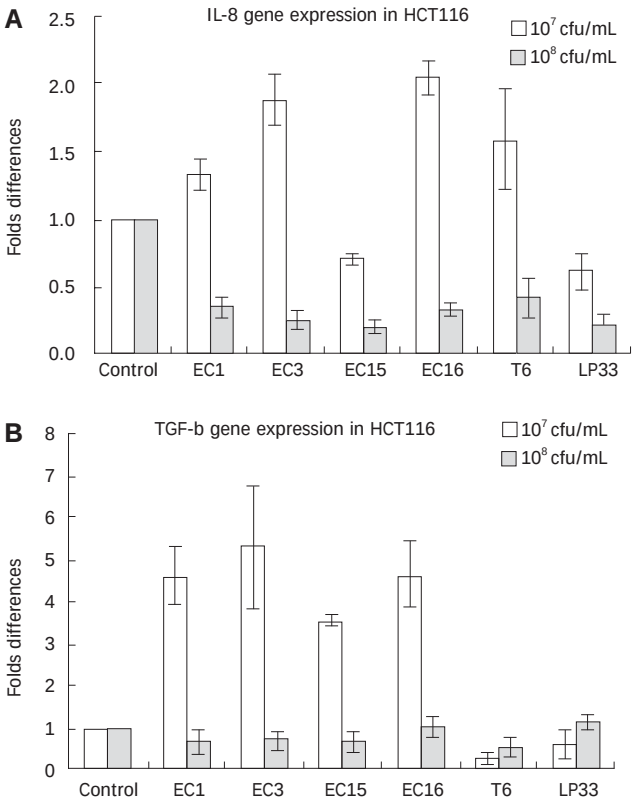


Figure 2 Different cytokines expression in HCT116 cells. RT-PCR analysis was used to determine IL-8 (**A**) and TGF-β (**B**) expression. 10^7 and 10^8 cfu/mL *E. faecalis* (EC1, EC3, EC15, EC16), LP33 and T6 were treated with HCT116, mRNA expression of the cytokines were analyzed as described in Materials and Methods. Quantitative densitometry was carried out on EtBr-stained gels and the results from three independent experiments were compiled to produce the data shown (mean ± SD). The gel densities were normalized against β-actin.

suppression of IL-8 secretion in all the three IECs.

The balance between inflammatory and regulatory cytokines is of crucial importance in the gut immune modulation. TGF-β is an important inflammation regulatory cytokine. In our study, TGF-β cytokine were greatly induced by *E. faecalis* (EC3, EC15) (10^7 cfu/mL) in Caco-2 cells (data not shown). Other LAB, however, could not induce TGF-β cytokine secretion above the detection level (above 62.5 pg/mL) in IECs. IL-4 and IL-10 secretion were not above the detection level in our study (Data not shown).

Immune gene modulation in IECs

Not consistent with IL-8 secretion, addition of *E. faecalis* (EC1, EC3, EC15, EC16) and LP33 (10^7 cfu/mL) to HCT116 cells enhanced IL-8 mRNA expression (Figure 2A). However, 10^8 cfu/mL *E. faecalis* (EC1, EC3, EC16), LP33 and T6 strongly suppressed IL-8 mRNA expression in HCT116 cells (Figure 2A). Interestingly, both concentrations of LAB suppressed IL-8 secretion in HCT116 cells (Figure 1C). Almost the same was observed in Caco-2 cells: IL-8 mRNA was upregulated by 10^7 cfu/mL *E. faecalis* (EC1, EC3, EC15, EC16), L.GG and L.bul. However, there was no significant change in IL-8 mRNA expression with 10^8 cfu/mL LAB stimulation (Data not shown). As shown in Figure 2B, TGF-β gene expression was greatly enhanced by 10^7 cfu/mL *E. faecalis* (EC1, EC3, EC15, EC16) and T6 in HCT116 cells, however, 10^8 cfu/mL of LAB showed no regulation of TGF-β gene expressions.

Activation of signal transduction pathway by TLRs led to the induction of inflammatory cytokine and chemokine genes that function in host defenses and tumor progression. The ability of the intestinal cell lines in response to *E. faecalis* and other LAB at the level of TLRs mRNA expression was therefore assessed using semi-quantitative RT-PCR. Gene encoding TLR1, TLR2, TLR3, TLR4, TLR5, TLR9, TRAF6 and Tollip were analyzed.

Examination of the TLR9 gene expression showed that TLR9 mRNA expression was greatly inhibited by 10^8 cfu/mL *E. faecalis* (EC1, EC3, EC15, EC16), T6 and LP33 treatment in HCT116 cells (Figure 3). As shown in Figure 3, TLR3 mRNA expression was also greatly inhibited by 10^8 cfu/mL *E. faecalis* (EC1, EC15, EC16), T6 and LP33 in HCT116 cells. Most interestingly, TRAF6 gene expression in HCT116 cells was totally inhibited

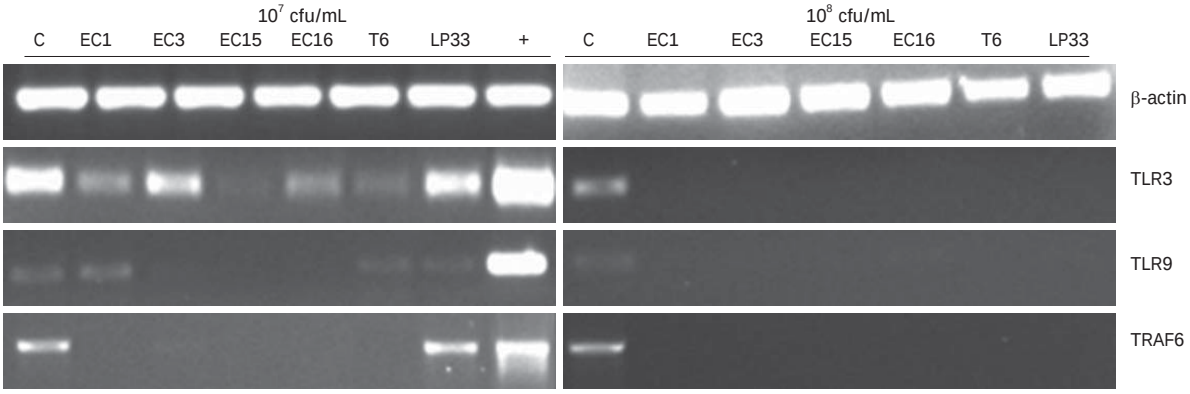


Figure 3 Induction of TLR3, TLR9 and TRAF6 mRNA expression in HCT116 cell line by *E. faecalis* (EC1, EC3, EC15, and EC16), T6, and LP33. The above LAB were added to HCT116 cells as described in Materials and Methods. RT-PCR analysis was used to determine their mRNA expression. "C" represents control using culture medium only. "+" is positive control in PCR reactions using specific plasmid.

Table 3 TLRs regulation in IECs upon the treatment of LAB

	TLR1	TLR3	TLR4	TLR5	TLR9	TRAF6	TOLLIP
HCT116	NE	S	NE	NE	S	S	NC
Caco-2	NE	NC	NC	NE	S	NC	NC
HT-29	NE	NC	S	NE	NC	S	NC

NE: No expression detected in the cells; NC: No significantly change upon the treatments; S: Gene was suppressed upon the treatments.

after treatment with 10^7 cfu/mL (except L.bul) and 10^8 cfu/mL LAB (Figure 3). In HT-29 cells, *E. faecalis* EC1, EC3 and EC15 but not EC16 suppressed TRAF6 mRNA expression (Data not shown). This observation parallels the TLR4 regulation in HT-29 cells. TLR4 is an essential receptor that transduces the signals of bacterial cell wall lipopolysaccharide (LPS). In the TLRs signaling pathways, TLR4 could activate numerous cytokine genes via TRAF6. TLR4 was not regulated significantly by LAB in Caco-2 cells. TLRs expression in these three cell lines were summarized in Table 3.

Possible effecter molecules that might be involved in the anti-inflammation study

Since some LAB tested, especially *E. faecalis*, inhibited IL-8 secretion in colon cancer cell lines, we further explored the possible effecter molecules that may play a role in the anti-inflammatory effects. For simplicity, we chose *E. faecalis* (EC1, EC3, EC15 and EC16), *L. rhamnosus* GG (L.GG) and *S. typhimurium* (Salm) for this study. The components from the bacteria were co-cultured with HCT116 cells. IL-8 level was measured using ELISA method.

First we tested the effects of sonicated bacteria and their cell wall components on IL-8 secretion. Cell wall components from all four *E. faecalis* strains demonstrated similar abilities as whole cells in reducing IL-8 levels (Data not shown). Cell debris of *S. typhimurium*, showed the expected ability in upregulating IL-8 levels. Co-culturing with *L. rhamnosus* GG cell debris caused no change in IL-8 concentration. To study whether attenuation of IL-8 level required bacterial cell contents, crude bacterial cell extract was co-cultured with HCT116 cells. In contrast to the results obtained using the cell debris, the crude cell extract of all four *E. faecalis* strains showed no ability in reducing IL-8 level (Data not shown).

Second, we tested the need for the four *E. faecalis* strains to be viable to attenuate IL-8 levels. The UV killed *E. faecalis* maintained its ability to suppress IL-8 levels (data not shown). The data implies that inhibition of IL-8 secretion from HCT116 cells does not need *E. faecalis* metabolites.

Third, to further confirm whether reduction in IL-8 levels required the direct contact between HCT116 cells and bacteria or could be mediated by a secreted product from either the bacteria or the intestinal epithelial cells, conditional media was made and added to HCT116 cells. In the first treatment (Section "Infection of intestinal cells"), supernatants of the controls containing IL-8 were harvested and used in the subsequent treatment as conditional media. It was shown that *E. faecalis* could not reduce the IL-8 level in the conditional media containing IL-8 (data not shown). Furthermore, intestinal epithelial cell cultures were incubated with bacteria housed in sterile plastic inserts, to ensure physical separation between the HCT116 cells and bacteria while allowing secreted bacterial products to reach the HCT116 cells. The results showed that all the bacterial strains could not suppress IL-8 levels as well (data not shown). Moreover, we investigated the possible ability of the four *E. faecalis* strains to stimulate a release of anti-inflammatory factors by HCT116 cells as described in the section "Conditional medium and cell inserts treatment", it was demonstrated that the supernatant harvested from co-culturing bacterial cells with HCT116 were incapable of reducing IL-8 levels in HCT116 cells.

Combining all the results, there is indication that the presence of bacterial cell wall components and attachment of bacteria to cells are essential in IL-8 attenuation.

To further figure out the possible molecules on the bacterial cell wall that played the key role in the attenuation of IL-8 secretion. The carbohydrates on bacterial cell walls were oxidized using m-periodate and iodate. Results of the four *E. faecalis* strains in buffer A showed an expected large decrease in IL-8 levels as seen in Figure 4A. Interestingly, the Na-iodate and Na-m-periodate treatments of them prevented an appreciable decrease in IL-8 levels (Figure 4A). Results from chymotrypsin and trypsin digested bacterial

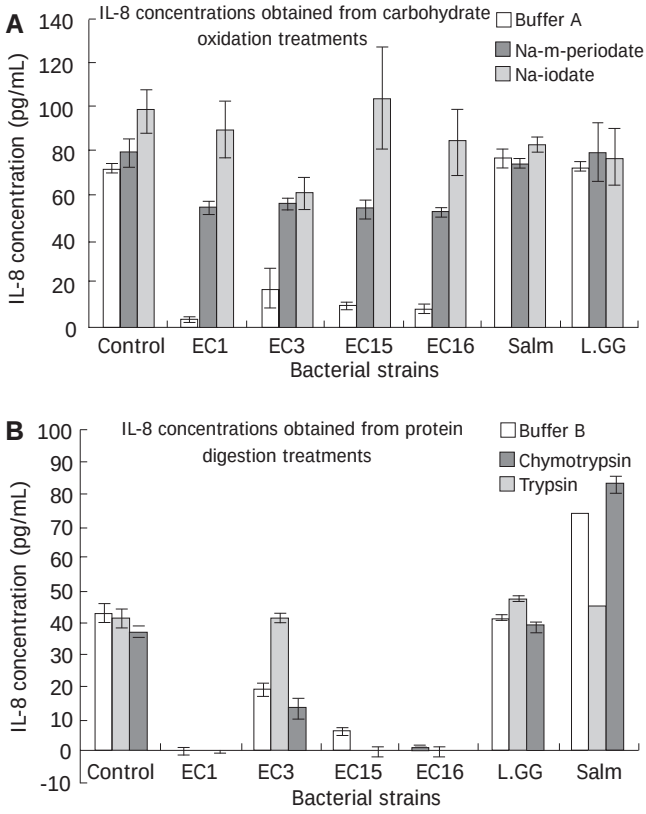


Figure 4 IL-8 secretion in HCT116 upon bacteria with carbohydrates oxidation and protein digestion. **A**: IL-8 concentrations obtained in HCT116 from treatments using bacteria with oxidized cell wall carbohydrates; **B**: IL-8 concentrations obtained in HCT116 from treatments using chymotrypsin- and trypsin-digested bacterial cell wall proteins in buffer B. Experiments were repeated three times independently. Standard deviations were indicated as error bars.

cell wall proteins in buffer B showing that buffer B, chymotrypsin and trypsin did not change the abilities of the *E. faecalis* strains in attenuating IL-8 level (Figure 4B). However, chymotrypsin treated *E. faecalis* EC3 seemed unable to significantly reduce IL-8 levels as seen in Figure 4B. *S. typhimurium* treated with buffer B and with chymotrypsin in buffer B showed no change in its capacity to increase IL-8 levels. Surprisingly, *S. typhimurium* treated with trypsin in buffer B showed a heightened ability in increasing IL-8 concentrations. These results suggest that carbohydrates on the bacterial cell wall are the major effecter molecules in the regulation of IL-8 secretion. However, proteins could also be involved.

In order to test which specific carbohydrates are important in reducing IL-8 levels, bacterial strains were treated with three different lectins. Succinyl-ConA and ConA have affinity for terminal α-D-glucosyl and α-D-mannosyl residues[21]. *Tetragonolobus purpureas* (TPA) on the other hand as an affinity for α-L-fucosyl residues and an especially high affinity for α-L-fucose residues on type Ⅱ chain blood group oligosaccharides[22]. These lectins would bind specifically to these residues and possibly change the overall carbohydrate structures, potentially affecting their role in attenuating IL-8 secretion. Figure 5 showed that the three lectins used also had no effect on depressing IL-8 secretion. Interestingly, with the employment of lectins, the ability to activate IL-8 by *S. typhimurium* was inhibited. This might imply that lectins may act on the carbohydrate in *S. typhimurium* to eliminate its stimulation on IL-8 secretion.

Adhesion assay

In order to study the importance of cell wall carbohydrates in adhering to HCT116 cells, carbohydrates on bacterial cell surfaces were oxidized using m-periodate. Figure 6 demonstrates that m-periodate treatment significantly reduced adhesion of all four *E. faecalis* strains to HCT116 cells (Table 4). Moreover, the secretion of IL-8 in HCT116 was greatly upregulated (Figure 4A). This study implies that carbohydrates on the bacterial cell wall helped bacteria to adhere to cells and the carbohydrate could act as ligands to activate signaling pathways to suppress IL-8 secretion from IECs. The carbohydrate could be structure component since sonicated and UV killed *E. faecalis* strains were able to suppress IL-8 secretion.

DISCUSSION

In the present study, we investigate the immune modulatory effects of infant intestinal *E. faecalis* compared with some other LABs on intestinal cells. Human intestinal cells (Caco-2, HT-29 and HCT116) have been shown to constitutively produce IL-8 in our study. IL-8, which is a potent neutrophil chemo-attractant and plays a key role in allergic inflammation, may be involved in the *E. faecalis* regulation of host immunology. The suppression of IL-8 secretion showed the reduction of inflammation reactions in animal and clinical trials[23]. In this study, most of the LABs tested, including most strains of *E. faecalis* (12 out of 16 strains isolated), did not show any effect on IL-8 production. However, IL-8 secretion was greatly downregulated after exposure to *E. faecalis* strains EC1, EC3, EC15, EC16 and some other LAB in IECs. This suggests that the immune reactions of an individual could be modulated by specific strains of a bacterial species, and his well being is determined by the specific strains of LAB which were established during early childhood. The inhibition of IL-8 protein secretion by *E. faecalis* could explain the increased incidence of allergy in infants deprived of *E. faecalis* in the intestine[6,8]. Although all 4 strains of *E. faecalis* and other LABs could inhibit the secretion of IL-8 (Figure 1), it is interesting to note that the different bacteria were reduced to a different level of IL-8 secretion after their exposure. *E. faecalis* in general suppressed IL-8 production to a higher degree than the other LABs. The downregulation of IL-8 secretion is dose dependent in Caco-2 and HCT116 cells. The higher the concentration of LAB, the less secretion of IL-8. Paradoxically, some of the IL-8 secretion was not parallel with its mRNA expression in the intestinal cell lines in response to the LAB. IL-8 mRNA was enhanced; however, its protein secretion was inhibited in IECs when they were exposed to *E. faecalis* at a concentration of 10^7 cfu/mL. More interestingly, IL-8 protein secretion was enhanced however its mRNA expression remained unchanged after the challenge of *S. typhimurium* at a concentration of 10^7 cfu/mL (data not shown). This implies that *E. faecalis* could boost the immune system to be ready for the challenge of pathogen at a physiological concentration

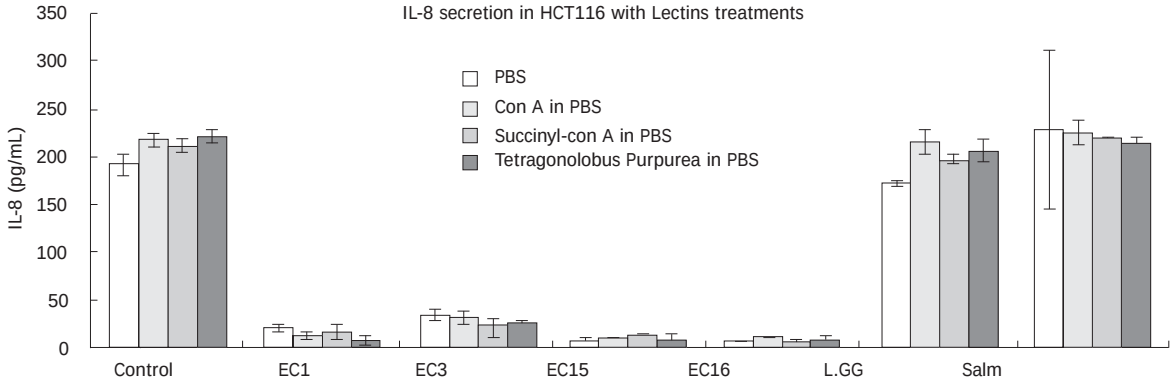


Figure 5 IL-8 secretion in HCT116 cells treated with three Lectins. Three lectins were added to HCT116 cells as described in Material and Methods. Experiments were repeated three times independently. Standard deviations were indicated as error bars.

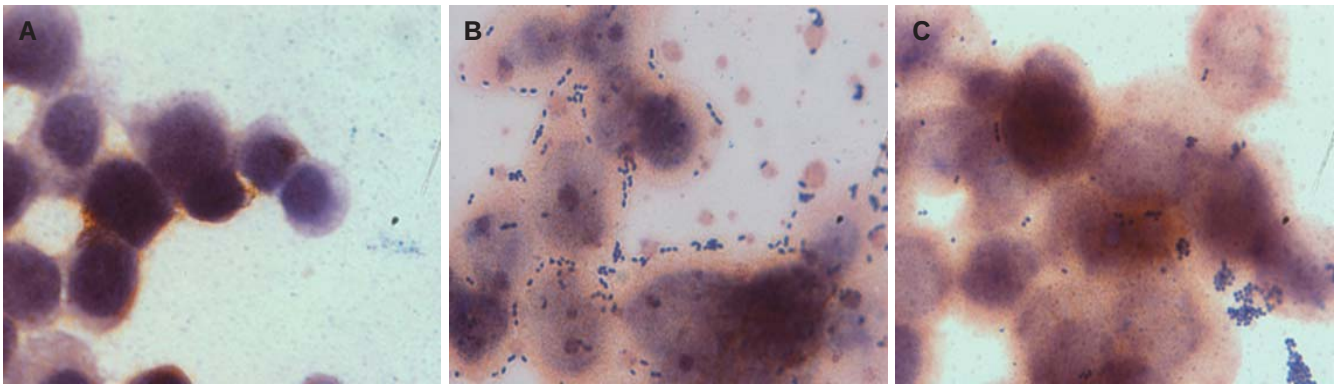


Figure 6 Monolayer of HCT116 cells with or without attached bacteria observed using light microscopy at 1000 ×. EC15 was used as a representation. **A**: Monolayer of HCT116 cells without bacteria treatment observed using light microscopy at 1000 ×; **B**: Monolayer of HCT116 cells with attached *E. faecalis* EC15 observed using light microscopy at 1000 ×; **C**: Monolayer of HCT116 cells with m-periodate treated *E. faecalis* EC15 observed using light microscopy at 1000 ×.

Table 4 Adhesion scores of *E. faecalis* after carbohydrate oxidation

Bacterial Strains	Adhesion score[1]		% change
	Whole cell bacteria	m-periodate treated bacteria	
E. faecalis EC1	1118.1	286	-74.42
E. faecalis EC3	1608	328	-79.6
E. faecalis EC15	1794	442.2	-75.35
E. faecalis EC16	693	282	-59.31
S. typhimurium[2]	49	40	-18.37
L. rhamnosus GG	605	850	40.5

[1]Adhesion is expressed as the number of attached bacteria/100 HCT116 cells, determined in 10 randomized microscope fields in three independent experiments. [2]Differences are not significant due to the small number of observed adherent bacteria.

(10^7 cfu/mL). However, the inflammatory responses were suppressed in the absence of pathogen and the presence of *E. faecalis*. On the contrary, *S. typhimurium* activated inflammatory responses without upregulating the immune gene expression. Thus, the regulation of IL-8 is important in the immune homeostasis. Its modulation by *E. faecalis* could involve post-transcriptional processes in protein synthesis and secretion[24]. The suppression of IL-8 at colon cancer cell lines' basal level could inhibit its inflammatory responses. Furthermore, these data suggests that IECs could differentiate commensal bacteria from pathogenic bacteria and thus lead to different immune responses.

Interestingly, TGF-β, which is important in inflammatory disease recovery, was upregulated significantly by *E. faecalis* EC3 and EC15 in Caco-2 cells at protein level and in HCT116 at the gene level. TGF-β is also a key factor implicated in the regulation of intestinal barrier function and is thought to mediate tolerance to the indigenous microflora. Compared with *Lactobacillus* and *Bifidobacterium*, *E. faecalis* demonstrated stronger effects on TGF-β production. The suppression of IL-8 secretion and activation of TGF-β production in the presence of *E. faecalis* suggested that *E. faecalis* could alter the balance among Th1 and Th2 immunities and leading to prevention of inflammation in intestinal cells.

Toll like receptors (TLRs) are also important in regulating inflammatory responses and Th1/Th2 immunities in the host. In our study, TLR3, TLR4, TLR9 were found to express in some of the colon cancer cell lines and their expression was suppressed by *E. faecalis*. Moreover, we found that TRAF6 mRNA was also suppressed by *E. faecalis* and some of the LABs in IECs. TRAF6 is an adaptor in TLRs signaling pathway. It could bind TAB1, TAK1 and TAB2 to form a complex leading to the activation of NF-κB and MAP kinases[25]. Thus, the suppression of TRAF6 could inhibit the activation of the NF-κB and the

MAPKs families and further the inflammatory responses. The inhibition of TLR3 and TLR9 signaling by LAB may explain the suppression of pro-inflammatory cytokines secretion in the colon cell lines. Hence, the suppression of TLR3 and TLR9 signaling could be used for therapeutic treatment of inflammatory diseases including cancer, asthma and allergy[26,27]. In addition, the low expression of TLR4 in IECs may explain the tolerance of the large quantities of luminal LPS in the intestine[28]. Furthermore, recent studies showed that bacteria-induced experimental colitis involves the activation of TLR[29]. The blocking of TLRs by *E. faecalis* may represent an attractive strategy to treat intestinal inflammation.

Overall, the modulation of intestinal immunity by *E. faecalis* may involve TRAF6, a number of TLRs (TLR3, TLR4, and TLR9) and cytokines (IL-8, TGF-β).

In order to characterize the effecter molecules that modulate inflammatory responses in IECs, studies were further carried out using conditional medium. The use of conditional medium showed that the molecules were not secreted by cells or bacteria or molecules activated after co-culturing the cells and bacteria. This finding was further confirmed using cell inserts. No IL-8 downregulation was observed when the cells and bacteria were separated by cell inserts. However, when bacterial cell wall fragments were applied to the intestinal cells after sonication, there was a dramatic suppression of IL-8 secretion in HCT116. In addition, the remaining supernatant, the crude cell extract containing bacterial cytoplasm, ribosomes, small membrane vesicles, and large membrane structures could not suppress IL-8 secretion. Therefore, the molecules are not inside the bacteria but on the surface of bacterial cell walls. Since only bacterial cell walls could inhibit IL-8 secretion, it implies that the inhibition does not involve bacterial metabolites. Another study using UV killed bacteria confirmed that those killed bacteria retained the ability to suppress IL-8 secretion in HCT116.

Results from oxidation of carbohydrate moieties on the whole cell surfaces of *E. faecalis* showed that the four *E. faecalis* lost their ability to depress IL-8 secretion. Thus, carbohydrates are the main effecter molecules on *E. faecalis*. This mild periodate oxidation at acid pH cleaves carbohydrate vicinal hydroxyl groups without changing polypeptide chain structures[30]. Periodate consumption and iodate formation, give insight to the number of vicinal dihydroxyl groups which possibly play the roles in suppression of inflammation[31]. Chymotrypsin-mediated protein digestion of the effecter molecules, on *E. faecalis* EC3's cell surface, stripped it of its ability to attenuate IL-8 levels. Thus *E. faecalis* EC3's effecter molecule probably has protein structure(s) necessary for depressing IL-8 secretion. Similar chymotrypsin-mediated protein digestion of the three other *E. faecalis* strains and trypsin-mediated protein digestion on all four *E. faecalis* strains failed to alter their ability in reducing IL-8 levels, suggesting that protein might not be the major components from the bacterial cell wall in suppressing IL-8 secretion.

The specific carbohydrate monosaccharides involved in IL-8 level attenuation could not be determined from the lectin study in our experiments. This study suggests that certain monosaccharides that are not part of the inflammation suppressing effecter molecule. α-L-fucosyl, α-L-fucose, D-mannosyl and D-glucosyl residues are probably not vital or possibly absent from the effecter molecule. However they might be important in upregulating IL-8 secretion in *S. typhimurium*.

The adhesion study of *E. faecalis* strains demonstrated that they adhered well to the mucosal layer of epithelial cells. Oxidation of the carbohydrate moieties on the bacterial cell surface caused a decrease in their adhesive abilities as well as a drop in the ability to suppress IL-8 secretion. This suggests that the cell surface carbohydrates are necessary for the adhesion of four *E. faecalis* strains to IECs. This is in congruence with Guzmàn *et al* who previously observed that enterococcal surface carbohydrate adhesins were necessary for binding of *E. faecalis* to Girardi heart human cells and urinary tract epithelial cells[32]. Other studies by Shorrock and Lambert also demonstrated that carbohydrate oxidation reduced the binding abilities of *E. faecalis*, suggesting that surface adhesin with the carbohydrate component is necessary for adhesion[33]. Since the same oxidation of carbohydrate moieties also stripped these enterococci of their IL-8 secretion suppressing abilities, adhesion of these bacteria *via* carbohydrate moieties plays a vital role in suppressing inflammation.

In conclusion, the study has demonstrated that *E. faecalis* is the major human intestinal LAB in modulating expression of host immune genes that participate in inflammation. Moreover, the immune regulatory mechanism may involve the adhesion of *E. faecalis* on intestinal cells and suppression of specific TLRs signaling pathways *via* carbohydrate mediated adhesion-receptor interaction.

ACKNOWLEDGMENTS

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COMMENTS

Background

It has long been assumed that indigenous intestinal microbiotas are involved in the immunomodulation of the host and the effects are strain dependent. However, there are few direct evidences in support of the assumption. In addition, the underlying molecular and cellular mechanisms are unclear.

Research frontiers

Recent studies showed that *Enterococcus faecalis* could suppress the proliferation of intestinal pathogens and thus may prevent infection and induction of inflammation. Studies on *Bacteroides thetaiotaomicron*, a prevalent commensal bacterium of the human intestine showed that it might attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-γ and Rel A. Toll like receptors (TLRs) and cytokines are involved in the anti-inflammation process.

Innovations and breakthroughs

In our knowledge, this is the first time *E.faecalis* is demonstrated as the main immune modulator in the intestinal Lactic acid bacteria (LAB) of infants, and cell wall carbohydrate is involved in the regulatory effects.

Applications

This finding highlights possible new approaches for therapeutic treatment of inflammatory disease in humans. The study suggests that the immunological status in the gastrointestinal tract of infants may be determined by the presence of specific strains of *E. faecalis*.

Terminology

Probiotics: Live microorganisms which can when administered in adequate amounts confer a health benefit on the host; LAB: Lactic acid bacteria comprise a clade of Gram positive, low-GC, acid tolerant, non-sporulating, non-respiring rod or cocci that are associated by their common metabolic and physiological characteristics. LAB is the most common type of microbe used as a probiotic.

Peer review

Wang *et al* showed that some Enterococcus strains reduced IL-8 secretion from intestinal epithelial cells. Focus of this manuscript is interesting.

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BASIC RESEARCH

Sources of calcium in agonist-induced contraction of rat distal colon smooth muscle *in vitro*

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Abstract

AIM: To study the origin of calcium necessary for agonist-induced contraction of the distal colon in rats.

METHODS: The change in intracellular calcium concentration ($[Ca^{2+}]_i$) evoked by elevating external Ca^{2+} was detected by fura 2/AM fluorescence. Contractile activity was measured with a force displacement transducer. Tension was continuously monitored and recorded using a Powerlab 4/25T data acquisition system with an ML110 bridge bioelectric physiographic amplifier.

RESULTS: Store depletion induced Ca^{2+} influx had an effect on $[Ca^{2+}]_i$. In nominally Ca^{2+}-free medium, the sarco-endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (1 μmol/L) increased $[Ca^{2+}]_i$ from 68 to 241 nmol/L, and to 458 ($P < 0.01$) and 1006 nmol/L ($P < 0.01$), respectively, when 1.5 mmol/L and 3.0 mmol/L extracellular Ca^{2+} was reintroduced. Furthermore, the change in $[Ca^{2+}]_i$ was observed with verapamil (5 μmol/L), La^{3+} (1 mmol/L) or KCl (40 mmol/L) in the bathing solution. These channels were sensitive to La^{3+} ($P < 0.01$), insensitive to verapamil, and voltage independent. In isolated distal colons we found that in normal Krebs solution, contraction induced by acetylcholine (ACh) was partially inhibited by verapamil, and the inhibitory rate was 41% ($P < 0.05$). On the other hand, in Ca^{2+}-free Krebs solution, ACh induced transient contraction due to Ca^{2+} release from the intracellular stores. The transient contraction lasted until the Ca^{2+} store was depleted. Restoration of extracellular Ca^{2+} in the presence of atropine produced contraction, mainly due to Ca^{2+} influx. Such contraction was not inhibited by verapamil, but was decreased by La^{3+} (50 μmol/L) from 0.96 to 0.72 g ($P < 0.01$).

CONCLUSION: The predominant source of activator Ca^{2+} for the contractile response to agonist is extracellular Ca^{2+}, and intracellular Ca^{2+} has little role to play in mediating excitation-contraction coupling by agonists in rat distal colon smooth muscle *in vitro*. The influx of extracellular Ca^{2+} is mainly mediated through voltage-, receptor- and store-operated Ca^{2+} channels, which can be used as an alternative to develop new drugs targeted on the dysfunction of digestive tract motility.

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Key words: Distal colon; Source of Ca^{2+}; Store-operated Ca^{2+} channel; Voltage-operated Ca^{2+} channel; Acetylcholine

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INTRODUCTION

Ca^{2+} is involved in many cellular transduction pathways, and regulation of different cellular phenotypes by $[Ca^{2+}]_i$ has been observed in many cell systems, including smooth muscle cells (SMC). Various agonists induce contraction in a variety of smooth muscle preparations, with a concomitant increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)[1,2]. The source for this ion is extracellular Ca^{2+} influx and Ca^{2+} release from intracellular stores. The relative contribution of intracellular and extracellular sources of Ca^{2+} depends on the tissue and mode of stimulation[3]. Moreover, agonist-induced increases in the cytosolic Ca^{2+} concentration produce smooth muscle contraction, characterized by a fast initial peak, the phasic component, followed by a decline to a lower maintained tension level, the tonic component[4,5].

In the former, we think two different Ca^{2+} channels participate in extracellular Ca^{2+} influx: voltage-operated Ca^{2+} channels (VOCCs) and receptor-operated Ca^{2+} channels (ROCCs)[6,7]. Participation of these channels in contraction varies among the types of smooth muscle present, the

agonists used, and the experimental conditions planned.

In addition to these influx pathways, there is now significant data from different cell types that demonstrate controlled Ca^{2+} influx also occurs in response to depletion of intracellular Ca^{2+} stores. We call these store-operated Ca^{2+} channels (SOCCs) or capacitative Ca^{2+} entry (CCE) channels. Previous studies have suggested SOCCs are restricted in non-excitable cells[8,9], but they have only recently been investigated in excitable cells , such as smooth muscle cells and neurons, which are involved in smooth muscle tone and regulation of synaptic plasticity. It has been reported there is a relationship between CCE and the regulation of smooth muscle tone with respect to their involvement in pulmonary, stomach, bronchial, vascular, gallbladder, and basilar artery tissue[10-15]. There is thus far no data to show that CCE are necessary as a source of Ca^{2+} in the smooth muscle of the rat distal colon.

The neurotransmitter acetylcholine (ACh) induces smooth muscle contraction *via* activating muscarinic receptors. ACh has been shown to elicit contraction that consists of a rapid phasic phase, followed by a tonic phase, by activation of M_3 muscarinic receptors in the rat and guinea pig gastric fundus and ileum, and the human ileum[16-19]. However, there have been some different results concerning the source of Ca^{2+} for muscarinic receptor-mediated contraction. It has been postulated that the sources of Ca^{2+} for phasic and tonic contraction induced by activation of muscarinic receptors differ among regions of the digestive tract and species of animals used.

It has been suggested that pharmacological manipulation of Ca^{2+} channels may be therapeutically useful in treating colonic motility disorders. Therefore, we tried to clarify the source of Ca^{2+} in the contraction induced by ACh. Sources of activator Ca^{2+} for muscarinic-mediated contraction of proximal colonic smooth muscle in rats have been well documented. With respect to the rat distal colon, the relative importance of the contribution of the different Ca^{2+} influx mechanisms, and the release of Ca^{2+} from the sarcoplasmic reticulum (SR), has not been assessed. The aim of this study was to evaluate the contribution of VOCCs, non-VOCCs and intracellular Ca^{2+} release to ACh-induced contraction in the smooth muscle of rat distal colon *in vitro*.

MATERIALS AND METHODS

Tissue preparation

A segment of distal colon (about 2 cm) was removed from Sprague-Dawley rats (150-250 g, supplied by the animal center of Anhui Medical University, China) killed by cervical dislocation. Pieces of colon were cleaned in a Petri dish, by perfusing with Krebs solution (114.0 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$, 1.8 mmol/L NaH_2PO_4, 11.5 mmol/L glucose, 18.0 mmol/L $NaHCO_3$, pH 7.4). The adipose and connective tissues were carefully stripped off with fine forceps. The endothelium of the colon was removed by gently rubbing the inner lumen of the vessels with a rough wooden stick.

Tension measurement

Pieces of distal colon were mounted in 40-mL organ baths containing Krebs solution, maintained at 37℃ and gassed with 95% O_2 and 50% CO_2, pH 7.4. One end of each piece was fixed to a hook on the bottom of the bath and the other attached to a force displacement transducer (Xinhang, Beijing, China). A resting preload of 1 g was applied to each colon, which was then allowed to equilibrate for 1 h. During this time, the Krebs solution was changed every 20 min. Tension was continuously monitored and recorded using a Powerlab 4/25T data acquisition system (Australia AD Instruments) with an ML110 bridge bioelectric physiographic amplifier (Australia AD Instruments). ACh and atropine were dissolved in the perfusate on the day of use.

Cell preparation

A segment of the distal colon was removed and placed in oxygenated HEPES/Ringer buffer solution (126 mmol/L NaCl, 6 mmol/L KCl, 6 mmol/L HEPES, 11 mmol/L $MgCl_2$, 1.5 mmol/L $CaCl_2$), pH 7.4 with 5 mol/L NaOH. The fecal contents were removed by repeated rinsing with HEPES/Ringer solution. After the segment was cut open longitudinally and pinned out in a dissection dish. Under a microscope, longitudinal and circular muscle layers were freed of the mesentery, serosa and mucosa, and cut into small pieces (2 mm^2) and rinsed in HEPES/Ringer solution for 20 min (at 37℃). Dispersion of smooth muscle cells was accomplished by three incubations at 31℃ with agitation in low-Ca^{2+} HEPES/Ringer solution containing collagenase type I (0.6 mg/mL), bovine serum albumin (2 mg/mL) and soybean trypsin inhibitor (0.6 mg/mL). They were then suspended in enzyme-free low-Ca^{2+} HEPES/Ringer solution and gently agitated through the tip of a wide-bore glass pipette to liberate single smooth muscle cells. Isolated cells were collected by low-speed centrifugation and resuspended in HEPES/Ringer solution. Trypan blue exclusion was used to verify cell viability (> 90% of all cells).

Measurement of $[Ca^{2+}]_i$ in distal colon smooth muscle cells

Measurement of $[Ca^{2+}]_i$ in colonic smooth muscle cells using fura-2 was performed using dual excitation wavelength fluorescence microscopy. Aliquots of cell suspension in HEPES/Ringer solution (10^6 cells/mL) were placed in Quartz cuvettes and incubated with 5 μmol/L fura-2/AM at 37℃ for 45 min. After being loaded with fura-2/AM, the cells were rinsed with Ca^{2+}-free solution, centrifuged (1500 r/min) and suspended in Ca^{2+}-free solution for the experiments that were carried out in a 960 MC spectrofluorophotometer (Shanghai Precision & Scientific Instruments, Shanghai, China). Cells were alternately illuminated at 340 and 380 nm and the intensity of fluorescence emission was measured at 500 nm. The $[Ca^{2+}]_i$ was calculated according to Grynkiewicz's equation: $[Ca^{2+}]_i = Kd\ [(F - F_{min})/(F_{max} - F)]$: where the dissociation constant Kd was 224 nmol/L at 37℃; F was fluorescence intensity under different experiment conditions; F_{max} was the maximum fluorescence intensity obtained in the presence of 0.1% Triton ×-100; and the minimum value (F_{min}) was obtained by using 5 mmol/L EGTA in the external medium. Before calculation, the autofluorescence measured from cells that unloaded

Table 1 Measurement of $[Ca^{2+}]_i$ in freshly dispersed suspension of smooth muscle cells from rat distal colon

Group	$[Ca^{2+}]_i$ (nmol/L)		
	Ca^{2+}-free medium	1.5 mmol/L $CaCl_2$ medium	3.0 mmol/L $CaCl_2$ medium
Control	68.32 ± 3.43	104.81 ± 6.37	194.44 ± 3.91
Thapsigargin	240.85 ± 12.65	457.55 ± 19.80[b]	1005.93 ± 54.62[b]

[b]$P < 0.01$ *vs* control.

fura-2/AM should be subtracted. All the measurements were finished in 40 min.

Drugs and solutions

Reagents were procured from the following vendors: thapsigargin, DMSO, ACh, atropine, lanthanum, fura-2/AM, Triton ×-100, EGTA, collagenase type I , bovine serum albumin (BSA) and verapamil were from Sigma (USA), and soybean trypsin inhibitor was from GIBCO (USA). The solutions of thapsigargin and fura-2/AM were prepared by dilution with DMSO. The concentration of DMSO in the final solution was < 0.1% and did not affect $[Ca^{2+}]_i$. In cell experiments, the Ca^{2+}-free HEPES/Ringer solution was prepared without $CaCl_2$ and with addition of EGTA (0.2 mmol/L). The low-Ca^{2+} solution had the same composition, except $CaCl_2$ was 0.03 mmol/L.

Statistical analysis

Biostatistical analysis was performed using the SPSS 11.0 software package. All experiments were repeated at least three times. Results of multiple experiments are given as the mean ± SE. Unpaired Student's *t* test was used for statistical evaluation in two-group comparisons. $P < 0.05$ was accepted as statistically significant.

RESULTS

Establishment of SOCCs

We first established whether SOCCs were present in rat distal colon smooth muscle cells. As shown in Table 1, for the fura-2/AM loaded cells, the incubation in Ca^{2+} free solution lasted 3 min, followed by the addition of two concentrations of Ca^{2+} (1.5, 3.0 mmol/L). It can be seen that cytosolic calcium rose from 68.32 in Ca^{2+}-free solution to 104.81 and 194.44 nmol/L for the two concentrations of 1.5 mmol/L and 3.0 mmol/L, respectively. The incubation of cells in Ca^{2+}-free solution in the presence of thapsigargin (1 μmol/L), followed by the addition of Ca^{2+}, 1.5 and 3.0 mmol/L, increased $[Ca^{2+}]_i$ that was significantly greater (455.77 and 1005.9 nmol/L) than that in the absence of thapsigargin.

Sensitivity to verapamil

To distinguish SOCC-mediated Ca^{2+} influx from that *via* L-type Ca^{2+} channels, we conducted experiments in a separate set of cells using 5 μmol/L verapamil. In these cells pretreated with verapamil, the $[Ca^{2+}]_i$ response to rapid reintroduction of $[Ca^{2+}]_o$ in the presence of thapsigargin ($n = 7$) was determined as above. We found the presence of verapamil during rapid reintroduction of

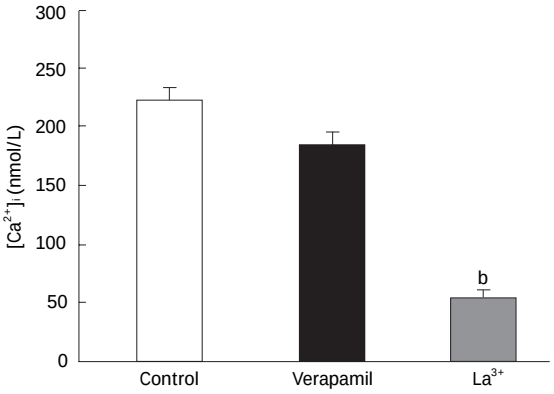


Figure 1 Ca^{2+} influx through SOCCs in enyzmatically dissociated smooth muscle cells of rats distal colon. After depletion of the SR by thapsigargin in the absence of extracellular Ca^{2+}, subsequent rapid reintroduction of extracellular Ca^{2+} resulted in activation of CCE *via* SOCCs. CCE were insensitive to verapamil, but blocked by La^{3+}. [b]$P < 0.01$ *vs* control.

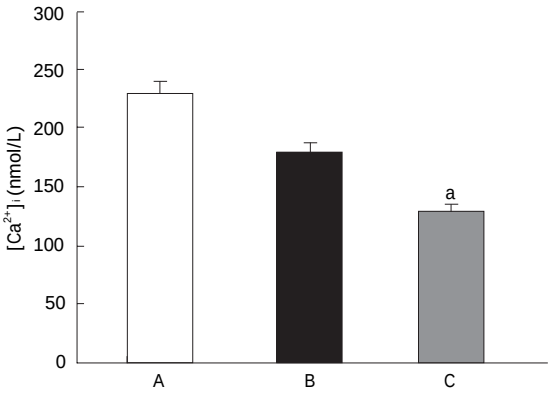


Figure 2 CCE are not mediated *via* L-type Ca^{2+} channels. (A) Controls; (B) 40 mmol/L K^+; (C) 60 mmol/L K^+. Only 60 mmol/L K^+ significantly decreased the Ca^{2+} influx mediated by CCE. [a]$P < 0.05$ *vs* control.

$[Ca^{2+}]_o$ (1.5 mmol/L) did not significantly influence Ca^{2+} influx induced by thapsigargin. Previous studies in other tissues have found that SOCC-mediated Ca^{2+} influx is inhibited by La^{3+}[20,21]. We investigated the sensitivity of multivalent cations to such Ca^{2+} influx. In a separate group of cells, pretreatment with La^{3+} and Ca^{2+} influx to rapid reintroduction in the presence of thapsigargin was greatly changed (Figure 1). Taken together, these results suggest the observed influx of extracellular Ca^{2+} in both instances was not mediated *via* L-type Ca^{2+} channels, but capacitative Ca^{2+} entry through SOCCs.

Role of membrane potential

A potential confounding factor in SOCC-mediated Ca^{2+} influx is the membrane potential, which alters the driving forces for Ca^{2+} entry. Accordingly, separate experiments were performed to determine the role of membrane potential. In the presence of verapamil (5 μmol/L), and pretreatment with high KCl solution (final concentrations of 40, 60 mmol/L), cells were re-exposed to thapsigargin, and $[Ca^{2+}]_o$ was then rapidly reintroduced (final concentration, 1.5 mmol/L). Figure 2 shows the presence of 40 mmol/L KCl did not significantly influence Ca^{2+} influx induced by

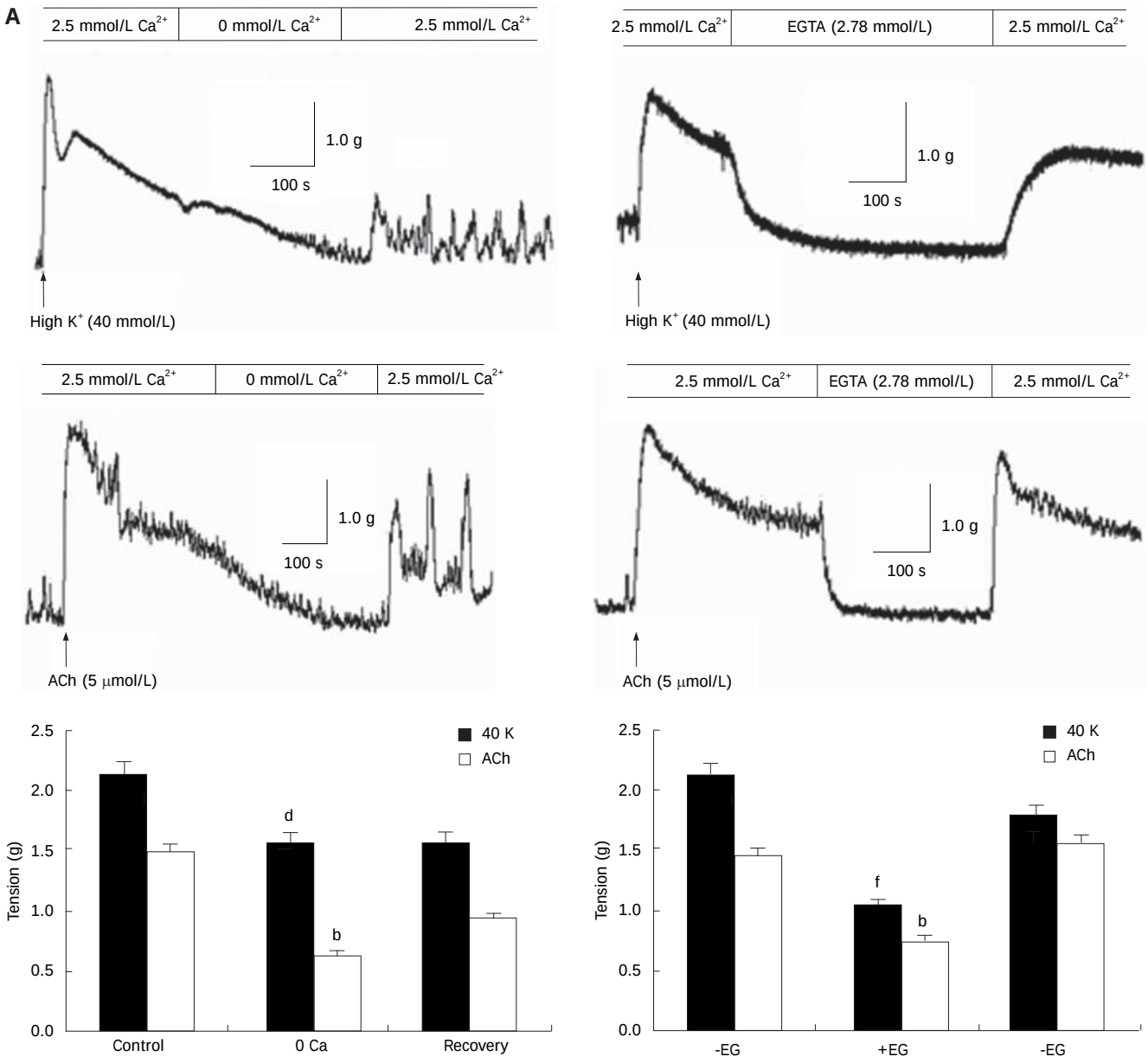


Figure 3 Role of Ca^{2+} in rat distal colon contraction. **A**: Representative results showing that removal of extracellular Ca^{2+} abolished contraction induced by high K^+ and 5 μmol/L ACh. Summarized data showing that tension induced by high K^+ and 5 μmol/L ACh before, during and after application of Ca^{2+}-free Krebs solution. $^bP < 0.01$, $^dP < 0.001$ *vs* Control; **B**: Representative results showing that chelation of extracellular Ca^{2+} with 2.78 mmol/L EGTA abolished contraction induced by high K^+ and 5 μmol/L ACh. Summarized data showing that tension induced by high K^+ and 5 μmol/L ACh in the presence of EGTA $^bP < 0.01$, $^fP < 0.001$ *vs*-EG (before).

thapsigargin. However, in the presence of 60 mmol/L KCl, Ca^{2+} influx was decreased ($P < 0.05$).

Requirement of extracellular Ca^{2+} for distal colon contraction

In isolated rat distal colon, removal of extracellular Ca^{2+} almost abolished the contraction induced by 40 mmol/L KCl or 5 μmol/L ACh, a muscarinic receptor agonist ($n = 11$, Figure 3A). Chelation of extracellular Ca^{2+} with 2.78 mmol/L EGTA, which decreases free Ca^{2+} concentration to 0.5 μmol/L in Krebs solution containing 2.5 mmol/L $CaCl_2$, had the same inhibitory effect on contraction induced by 40 mmol/L K^+ or 5 μmol/L ACh ($n = 7$, Figure 3B). Furthermore, extracellular application of the L-type VOCC blocker verapamil (5 μmol/L) almost abolished the 40 mmol/L K^+-induced tension. The tension decreased from 2.1 to 0.52 g and the inhibitory rate was 74% ($n = 7$, $P < 0.001$). The tension induced by 5 μmol/L ACh was also inhibited, but the inhibitory rate was only 41%. The tension decreased from 1.44 to 0.85 *g* ($n = 7$, $P < 0.05$) (Figure 4). These observations indicated ACh induced rat distal colon contraction by activating multiple Ca^{2+} entry pathways, whereas 40 mmol/L K^+-induced rat distal colon contraction was solely dependent on the membrane-depolarization-mediated opening of VOCCs.

CCE-mediated distal colon contraction

In the absence of extracellular Ca^{2+}, ACh induced a transient contraction that was apparently due to Ca^{2+} release from the SR. The muscarinic receptor blocker atropine (10 μmol/L) was applied to the distal colon when the ACh-induced contraction returned to baseline.

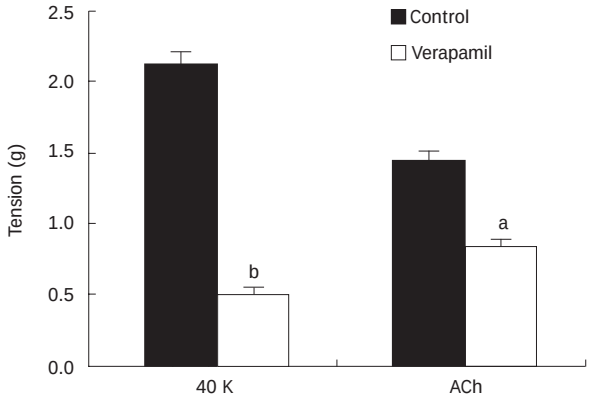


Figure 4 Effects of the VOCC blocker verapamil on distal colon contraction induced by high K^+ and ACh. $^aP < 0.05$, $^bP < 0.001$ *vs* control.

In the presence of atropine, restoration of extracellular Ca^{2+} caused tonic contraction (Figure 5Aa). We further showed tonic contraction was unaffected by verapamil ($n = 5$, Figure 5Ab). The CCE-mediated contraction in the presence of atropine was about 0.46-fold greater than that induced by SR Ca^{2+} release and the total peak contraction induced by ACh in the presence of extracellular Ca^{2+} ($n = 9$, Figure 5B). Taken together, these results showed that Ca^{2+} release from the SR and CCE *via* SOCCs both contribute to agonist-mediated distal colon contraction.

Inhibitory effects of La^{3+} on CCE-mediated distal colon contraction

La^{3+} has been shown to block SOCCs in many cell types[11,31]. La^{3+} (50 μmol/L) was applied to the distal colon when CCE-induced contraction was maximized, and the CCE-mediated contraction was significantly attenuated ($n = 7$, Figure 6A and B). These results indicated the properties of SOCCs in the distal colon are similar to those found in other tissues.

DISCUSSION

The results of this study reveal smooth muscle cells of rat distal colon express functional SOCCs, which play a vital role in agonist-induced contraction.

In many cells, depletion of intracellular Ca^{2+} stores is coupled to activation of CCE. The CCE model has gained support from experiments that used inhibitors of sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase (SERCA), such as thapsigargin[22,23]. These drugs cause passive store depletion of Ca^{2+} by blocking the reuptake of Ca^{2+} into the SR storage pools[24,25]. Studies in a variety of preparations have demonstrated that SERCA inhibitors increase Ca^{2+} influx and/or smooth muscle tone[26,27]. To investigate the role of CCE in the contractile response in rat distal colon smooth muscle, freshly dissociated cells were incubated with thapsigargin in Ca^{2+}-free HEPES/Ringer solution, followed by reintroduction of extracellular Ca^{2+}, which greatly elevated $[Ca^{2+}]_i$. The lack of effect of thapsigargin resulted in only a slightly elevated $[Ca^{2+}]_i$. Moreover, the properties of the pathway were consistent with the CCE through SOCC found in several types of non-excitable cells. They are insensitive to verapamil

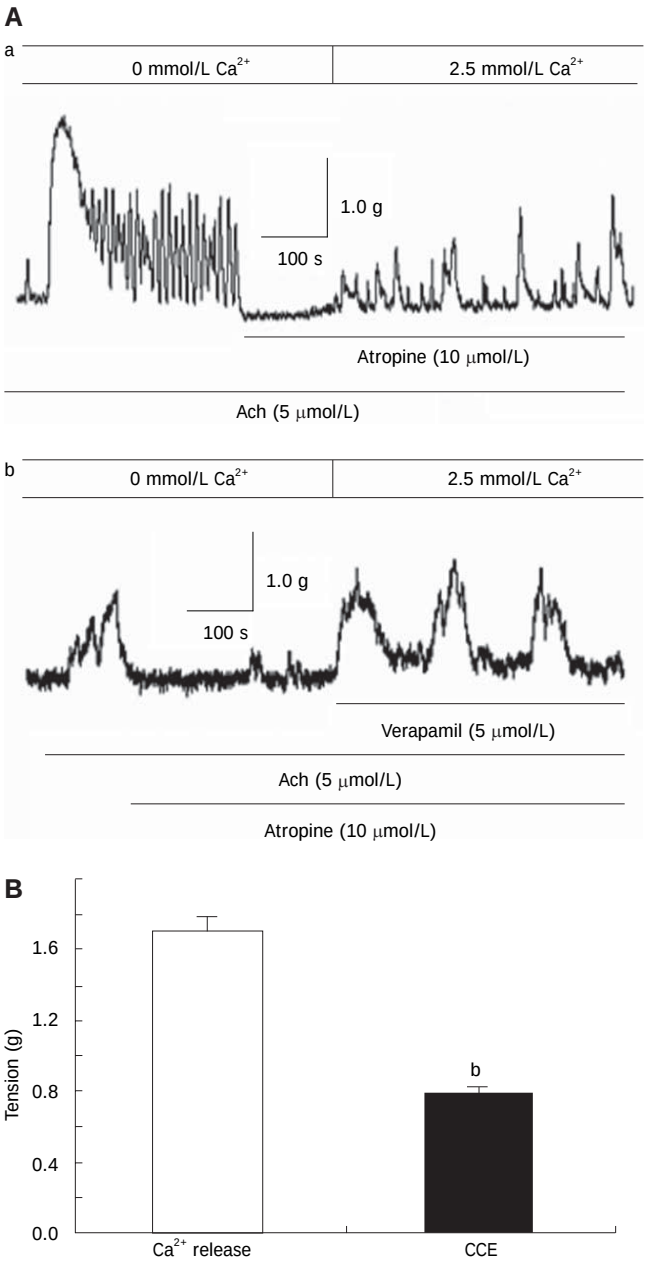


Figure 5 Contribution of Ca^{2+} release from SR stores and Ca^{2+} influx *via* CCE in ACh-induced distal colon contraction. **A**a: In the presence of atropine, restoration of extracellular Ca^{2+} induced contraction, most likely due to Ca^{2+} influx *via* CCE; **A**b: VOCC blocker verapamil (5 μmol/L) negligibly affected CCE-mediated contraction in the presence of atropine; **B**: Summarized data showing that CCE-mediated contraction is about 0.46-fold greater than the contraction induced by SR Ca^{2+} release in ACh-induced rat distal colon contraction. $^bP < 0.001$ *vs* Ca^{2+} release.

(L-type VOCC inhibitor), but sensitive to La^{3+} (SOCC inhibitor). Unlike VOCCs, SOCCs do not appear to be greatly affected by changes in membrane potential. If thapsigargin-induced elevation of $[Ca^{2+}]_i$ in the presence of verapamil is mediated *via* VOCCs, most positive membrane potentials should increase Ca^{2+} influx. However, in this study, such Ca^{2+} influx was not significantly altered by 40 mmol/L K^+. Nevertheless, we do not rule out the role of VOCCs in distal colon smooth muscle cells, because 60 mmol/L K^+ greatly altered elevation of $[Ca^{2+}]_i$. The relative contribution of VOCCs and SOCCs depends on whether a specific agonist causes membrane potential depolarization and/or only intracellular Ca^{2+} release.

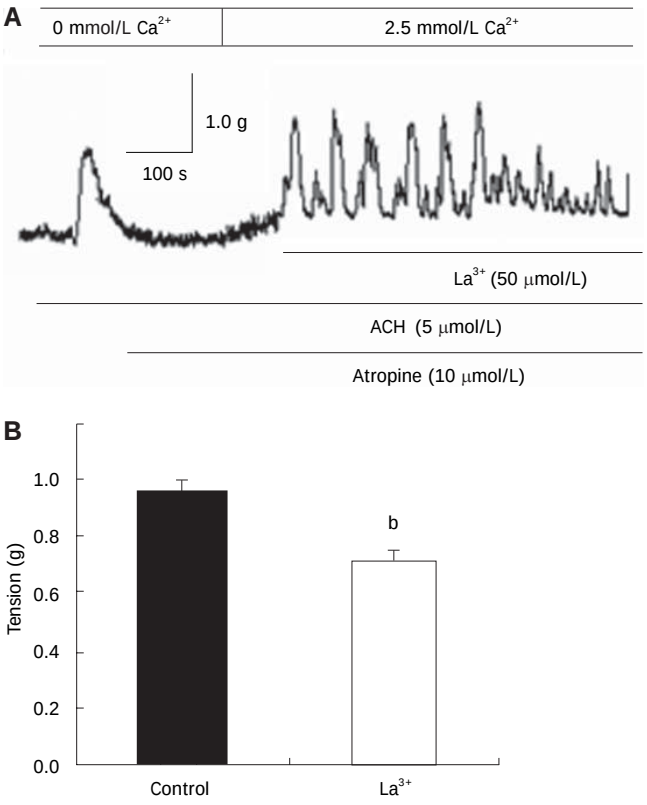


Figure 6 Inhibitory effects of La^{3+} on CCE-mediated distal colon contraction. $^bP < 0.01$ *vs* control. Control: CCE-mediated contraction.

In the rat distal colon, ACh and high K^+ induced the contraction of smooth muscle in a phasic and tonic phase. It has been suggested the phasic contraction is induced by Ca^{2+} released from the SR, whereas the tonic phase is induced by Ca^{2+} that enters from outside the cell[28]. Both pathways for inducing contraction were found in the rat distal colon in the present study. The tonic phase was abolished or significantly inhibited by removal of extracellular Ca^{2+} or chelation of extracellular Ca^{2+} with 2.78 mmol/L EGTA. The present results suggest extracellular Ca^{2+} is necessary for the contraction induced by high K^+ and ACh. ACh studied in the rat distal colon activates Ca^{2+} influx, which may be mediated by different mechanisms, from outside of the smooth muscle cells.

Having established in the present study that the contractile response to ACh in rat distal colon smooth muscle is dependent on extracellular Ca^{2+} influx, we then investigated the contributions of VOCCs and non-VOCCs to this response. Tissue pretreatment with verapamil essentially abolished the 40 mmol/L K^+-induced contraction, but only partially attenuated the response to 5 μmol/L ACh, and the inhibitory rate was 41%. These results indicate the contraction induced by high K^+ is mainly dependent on Ca^{2+} release from SR stores and Ca^{2+} influx mediated by VOCCs. Ca^{2+} influx mediated *via* VOCCs and non-VOCCs is most important for the contraction of rat distal colon smooth muscle induced by ACh.

The muscarinic receptor plays a key role in the parasympathetic nervous control of various peripheral tissues including the gastrointestinal tract[29]. Pharmacological characterization of the contractile response to ACh has suggested the $M_3/G_{q/11}/PLC\beta$ pathway, which acts through the production and release of inositol 1,4,5-trisphosphate (IP_3) to produce mobilization of stored Ca^{2+}, is largely responsible for the contraction[28]. Rather, it is believed the depletion of intracellular Ca^{2+} stores by IP_3 generates an as-yet-uncharacterized signal that activates CCE[30,31]. Because in the present study, ACh induced transient contraction that was apparently due to Ca^{2+} release from the SR in the absence of extracellular Ca^{2+}, the question arises, does CCE has any role in distal colon motility? We found that after the ACh-mediated transient contraction in the absence of extracellular Ca^{2+} and the return to baseline, the subsequent restoration of extracellular Ca^{2+} induced a sustained contraction in the presence of the muscarinic-receptor blocker atropine. Such sustained contraction was not sensitive to verapamil, and was decreased by 50 μmol/L La^{3+}. This contraction was independent of: (1) activation of the muscarinic receptor, (2) intact endothelium, and (3) opening of VOCCs. The result indicated that in the absence of extracellular Ca^{2+}, ACh actively depleted Ca^{2+} from the SR by increasing IP_3 production; therefore, activation of SOCCs and induction of CCE may be the trigger for this contraction. Taken together, the results shows that Ca^{2+} release from the SR is responsible for the phasic contraction, and that CCE *via* SOCC is necessary for the sustained contraction of ACh-induced rat distal colon contraction.

In summary, this study demonstrated the predominant source of activator Ca^{2+} for the contractile response to ACh is extracellular Ca^{2+}, and that intracellular Ca^{2+} has little role to play in mediating excitation-contraction coupling by ACh in rat distal colon smooth muscle *in vitro*. Influx of extracellular Ca^{2+} is mainly mediated by VOCCs, ROCCs and SOCCs. The study of Takeuchi *et al*[32] has shown that the source of Ca^{2+} in smooth muscle cells in the guinea pig proximal colon is mainly dependent on VOCCs. It is postulated that the source of activator Ca^{2+} for the contractile response varies among different regions of the gastrointestinal tract and the animals used. Further studies will hopefully be helpful in developing new drugs with unique properties, such as those that inhibit CCE mediated Ca^{2+} influx.

COMMENTS

Background

Various agonists induce contraction in a variety of smooth muscle preparations, with a concomitant increase in $[Ca^{2+}]_i$. It is suggested the sources of Ca^{2+} for phasic and tonic contraction induced by activation of muscarinic receptors differ among regions of the digestive tract and species of animals used.

Research frontiers

Intracellular Ca^{2+} is a critical second messenger responsible for linking external stimuli to contraction, proliferation and gene expression. It has been suggested some of the motor changes associated with intestinal infection, stress and inflammation are associated with alterations in $[Ca^{2+}]$. Further studies of pharmacological manipulation of Ca^{2+} channels may be therapeutically useful in treating colonic motility disorders.

Innovations and breakthroughs

In this study, we investigated the importance of Ca^{2+} influx in smooth muscle contraction. We also evaluated the contribution of the different Ca^{2+} influx mechanisms and the release of Ca^{2+} from the sarcoplasmic reticulum (SR) to

contraction induced by activation of muscarinic receptors in rat distal colon *in vitro*. The results showed that Ca^{2+} necessary for contractile response in the distal colon of rat was supplied from outside the smooth muscle cells via voltage-operated Ca^{2+} channels (VOCCs), receptor-operated Ca^{2+} channels (ROCCs) and store-operated Ca^{2+} channels (SOCCs), and the SR had a minor role in the contractile response.

Applications

The results provide further evidence for the contraction of the rat distal colon, which hopefully will be of importance in developing new drugs with unique properties, such as those that inhibit capacitative Ca^{2+} entry (CCE)-mediated Ca^{2+} influx.

Terminology

Thapsigargin is a potent skin irritating sesquiterpene lactone isolated from the roots of Thapsia garganica L. It also acts as a non-phorbol-ester-type tumor promoter, which discharges intracellular Ca^{2+} stores by specific inhibition of sarcoplasmic endoplasmic reticulum Ca2+-ATPase (SERCA).

Peer review

This paper examined the relative contributions of internal calcium release and calcium influx to phasic and tonic contraction in the distal colon, and measured calcium concentration and calcium influx in isolated colon smooth muscle cells. The work addressed an old topic but the current discovery of SOCCs in excitable cells such as smooth muscle cell (SMC) is interesting.

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RAPID COMMUNICATION

Factors associated with time to laparoscopic cholecystectomy for acute cholecystitis

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Abstract

AIM: To determine patient and process of care factors associated with performance of timely laparoscopic cholecystectomy for acute cholecystitis.

METHODS: A retrospective medical record review of 88 consecutive patients with acute cholecystitis was conducted. Data collected included demographic data, co-morbidities, symptoms and physical findings at presentation, laboratory and radiological investigations, length of stay, complications, and admission service (medical or surgical). Patients not undergoing cholecystectomy during this hospitalization were excluded from analysis. Hierarchical generalized linear models were constructed to assess the association of pre-operative diagnostic procedures, presenting signs, and admitting service with time to surgery.

RESULTS: Seventy cases met inclusion and exclusion criteria, among which 12 were admitted to the medical service and 58 to the surgical service. Mean ± SD time to surgery was 39.3 ± 43 h, with 87% of operations performed within 72 h of hospital arrival. In the adjusted models, longer time to surgery was associated with number of diagnostic studies and endoscopic retrograde cholangio-pancreatography (ERCP, $P = 0.01$) as well with admission to medical service without adjustment for ERCP ($P < 0.05$). Patients undergoing both magnetic resonance cholangiopancreatography (MRCP) and computed tomography (CT) scans experienced the longest waits for surgery. Patients admitted to the surgical versus medical service underwent surgery earlier (30.4 ± 34.9 *vs* 82.7 ± 55.1 h, $P < 0.01$), had less post-operative complications (12% *vs* 58%, $P < 0.01$), and shorter length of stay (4.3 ± 3.4 *vs* 8.1 ± 5.2 d, $P < 0.01$).

CONCLUSION: Admission to the medical service and performance of numerous diagnostic procedures, ERCP, or MRCP combined with CT scan were associated with longer time to surgery. Expeditious performance of ERCP and MRCP and admission of medically stable patients with suspected cholecystitis to the surgical service to speed up time to surgery should be considered.

Key words: Acute cholecystitis; Laparoscopic cholecystectomy; Endoscopic retrograde cholangiopancreatography; Post-operative complications

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INTRODUCTION

Nearly 700 000 cholecystectomies are performed in the United States annually, making it the most commonly performed operation on the digestive tract[1,2]. The optimal timing for the performance of laparoscopic cholecystectomy (LC) in the subset of patients with acute cholecystitis has been a point of much discussion. Initially there was concern about potential dangers of performing an operation on an acutely inflamed gallbladder with instruments that lacked the feedback, dexterity, and precision of human hands[3,4]. Studies within the past ten years, however, have determined that laparoscopic cholecystectomy for patients with acute cholecystitis within three days of admission is not only safe but also associated with fewer post-operative complications, decreased morbidity, decreased length of hospital stay, and decreased overall cost when compared to delayed or interval LC[5-9]. Cognizance of the general preferability of prompt surgery, however, may not be uniform among all caretakers. Hence,

we examined the time to laparoscopic cholecystectomy in our institution. In order to help identify potential areas for improvement in current management, we also examined the association of presenting signs, pre-operative imaging studies and procedures, and admitting service (medicine versus surgery) with time to operation.

MATERIALS AND METHODS

We performed a retrospective medical record review of patients identified by a primary or secondary discharge diagnosis of acute cholecystitis (ICD codes 575.0, 575.10, 575.11 and 575.12) at Norwalk Hospital between March 2003 and December 2005. The diagnosis of acute cholecystitis was made at the time of surgery by experienced biliary surgeons and confirmed by acute inflammatory changes being found on pathologic examination[10]. Patients not undergoing cholecystectomy during this hospitalization were excluded from analysis. Data collected included age, sex, symptoms and physical findings, laboratory and radiological investigations performed, co-morbidities, time from arrival to the hospital to operation, admission service, complications, and length of stay (LOS). Patients presenting with discharge diagnosis of acute cholecystitis but not undergoing cholecystectomy during the hospitalization were excluded.

In order to help account for the contributions of pre-operative comorbid illnesses to the measured outcomes, we classified co-morbidities as none, minor (abnormal electrocardiogram (ECG), rhythm other than sinus, low functional capacity, history of stroke, uncontrolled hypertension), intermediate (mild angina, previous myocardial infarction, compensated or prior heart failure, diabetes mellitus, renal insufficiency), or major (unstable coronary syndromes, decompensated heart failure, significant arrhythmias, severe valvular disease) following the criteria by American Heart Association/American College of Cardiology (AHA/ACC) for cardiovascular risk[11]. An abnormal ECG is one indicating left ventricular hypertrophy, left bundle branch block, ST-T abnormalities. Advanced age is also listed in the AHA/ACC criteria as a minor clinical predictor but is undefined. For this study, it was arbitrarily designated as > 75 years old. Liver cirrhosis, which is not included in the AHA/ACC criteria, was added as a major peri-operative risk factor because it is known to increase post-operative mortality[12,13].

Post-operative complications were defined as newly arising occurrences related to the patient's surgical disease or management that prolonged length of stay or required treatment. Complications were categorized as mild (e.g., urinary tract abnormalities, prolonged ileus, prolonged biliary drainage), moderate (e.g., systemic inflammatory response, deep wound infection), and severe (e.g., death, respiratory failure requiring intubation, myocardial infarction)[14].

Statistical analysis

Descriptive analysis was performed for baseline demographic and clinical characteristics. Because of the natural clustering of the observations within physicians/surgeons, we employed a hierarchical generalized linear modeling approach to compare the risk-adjusted mean differences in "time-to-operation" and to obtain the posterior distribution of the true effect size upon change of time by the design variables admission team, pre-operative diagnostic imaging studies, and select physical and laboratory signs indicative of cholecystitis or an inflammatory state (Murphy's sign, temperature > 37.8℃, WBC > 10 200/UL)[15]. Other physical signs indicative of cholecystitis were excluded from the analysis because of infrequency or lack of specificity. This statistical approach accounts for within-physician/surgeon correlation of the observed outcomes and separates within-physician/surgeon variation from between-physician/surgeon variation.

Our aim was not to develop a predictive model but rather to determine the influence of different values of design variables on the change in time-to-surgery with and without controlling for patient characteristics. Thus, the first model was fitted without adjusting for any patient characteristics. The second model adjusted for patient demographics, including age and gender, the third model adjusted for patient demographics, and co-morbidity. The 95% credible interval (CI) was also calculated for each estimate obtained from the models. All of the statistical analyses were conducted using STATA version 8.0 (STATA Corporation, College Station, TX) and SAS version 8.12 (SAS Institute Inc. Cary, NC).

RESULTS

A total of 88 patient charts with the discharge diagnosis of acute cholecystitis were reviewed. In six patients the pathology report did not confirm a diagnosis of acute cholecystitis, and it was concluded that they suffered from biliary colic. An additional 12 patients were discharged without cholecystectomy and were also felt to have had biliary colic rather than acute cholecystitis. These 18 patients were excluded from analysis, yielding a study population of 70 patients. The mean ± SD age was 53 ± 17.3 years. The majority of patients (69%) had no recognized peri-operative co-morbidities (Table 1). Among those with co-morbidities, 16% were classified as minor, 13% were intermediate, and 3% were major. All but one patient presented with abdominal pain and nearly all (89%) had right upper quadrant tenderness on examination. Murphy's sign, considered to be a relatively specific sign in cholecystitis, was present in only one-quarter of the patients. Fever (> 37.8℃ and leukocytosis), which together with Murphy's sign comprise the diagnostic triad for acute cholecystitis, was present in only 23% and 51% of patients, respectively. Guarding was noted in 11% and rebound tenderness was detected in one patient.

Eleven patients presented with aspartate aminotransferase (AST) and alanine aminotransferase (ALT) greater than three times the upper normal limit and 2 (18%) required pre-operative ERCP for stone extraction. Two other patients had pre-operative ERCP performed because of common bile duct dilatation identified on CT scan. All ERCP examinations were therapeutic, removing stones from the common bile duct via sphincterotomy. Three additional patients required post-operative ERCP for retained stones.

The majority of patients were admitted to the surgical

Table 1 Patient characteristics *n* (%)

	Medical service *n* = 12	Surgical service *n* = 58	Total *n* = 70	*P* value
Mean age (range)	66 (38-89)	52 (20-83)	53 (20-89)	< 0.05
Age > 75 yr	5 (42)	7 (12)	12 (17)	
Sex				
Male	5 (42)	25 (43)	30 (43)	
Female	7 (58)	33 (57)	40 (57)	
Co-morbidities				< 0.05
None	4 (33)	44 (76)	48 (69)	
Minor	4 (33)	7 (12)	11 (16)	
Abnormal ECG	8 (67)	16 (28)	24 (34)	
Rhythm other than sinus	5 (42)	2 (3)	7 (10)	
Poor functional capacity	5 (42)	4 (69)	9 (13)	
History of stroke	0 (0)	3 (5)	3 (4)	
Intermediate	4 (33)	5 (9)	9 (13)	
Prior MI	3 (25)	3 (5)	6 (8)	
Compensated or prior CHF	2 (17)	0 (0)	2 (3)	
Diabetes mellitus	2 (17)	5 (9)	7 (10)	
Major	0 (0)	2 (3)	2 (3)	
Liver cirrhosis	0 (0)	2 (3)	2 (3)	
Physical examinaiton				
WBC > 10.2	7 (58)	29 (50)	36 (51)	
Murphy's sign	4 (33)	15 (26)	19 (27)	
Temp > 37.8℃	2 (17)	14 (24)	16 (23)	

ECG: Electrocardiogram; MI: Myocardial infarction; CHF: Compensated heart failure.

Table 2 Hospital course *n* (%)

	Medical service *n* = 12	Surgical service *n* = 58	Total *n* = 70	*P* value
Tests done				
Ultrasound	9 (75)	44 (76)	53 (76)	
CT scan	9 (75)	24 (41)	33 (47)	< 0.05
HIDA scan	4 (33)	12 (21)	16 (23)	
MRI/MRCP	1 (8)	1 (2)	2 (3)	
ERCP	4 (33)	3 (5)	7 (10)	
Time to surgery				
Mean time to operation, h (SD)	82.7 (55.1)	30.4 (34.9)	39.3 (43.4)	< 0.01
Operation within 3 d	7 (58)	54 (93)	61 (87)	< 0.01
Operation within 1 d	2 (17)	34 (59)	36 (51)	< 0.01
Length of stay				
Mean, D (SD)	8.1 (5.2)	4.3 (3.4)	5.0 (3.9)	< 0.01
3 d or less	2 (17)	30 (52)	32 (46)	< 0.05
6 d or more	8 (67)	11 (19)	19 (27)	< 0.01
Conversion to open	1 (8)	3 (5)	4 (6)	
post-operative complications	7 (58)	7 (12)	14 (23)	< 0.01
Mild	4 (33)	5 (9)	9 (13)	
Moderate	2 (17)	3 (5)	5 (7)	
Severe	1 (8)	1 (2)	2 (3)	

HIDA: Nuclear Hepatobiliary scan; MRI: Magnetic resonance imaging ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography.

service (83%) (Tables 1 and 2). Patients admitted to the medical service were significantly older (66 years *vs* 52 years, $P < 0.05$), and had more minor and intermediate, but not severe, peri-operative cardiovascular risk factors ($P < 0.05$), including diabetes mellitus, prior heart failure, and age > 75 years. Other non-cardiovascular comorbid conditions among the patients admitted to the medical service included dementia, anxiety, brain tumor, hepatitis B, and chronic Clostridium difficile infection.

Patients admitted to the medical service had significantly more abdominal CT scans done in the evaluation process (Table 2). The distribution of other pre-operative tests was not significantly different between the surgical and medical services. There was a trend toward more medicine patients undergoing pre-operative ERCP, 2 of 12 (17%) patients *vs* 2 of 58 (3%) patients, though this did not reach statistical significance.

The mean time to operation from admission was 39.3 ± 43.1 h (Table 2). Operation within 72 h occurred in 87% of the cases and operation within 24 h occurred in 51% of the cases. Patients admitted to the surgical service compared to the medical service had a significantly decreased mean time to operation, and significantly more surgical patients were operated on within 3 d as well as within 24 h (Table 2).

Post-operative complications occurred in 16 cases (23%). Complications occurred more frequently in medicine patients: 7 of 12 (58%) *versus* 9 of 58 (16%) surgical patients, $P < 0.01$ (Table 2). Complications included prolonged (> 2 d) ileus ($n = 2$), discharge with Jackson-Pratt drain for biliary drainage ($n = 2$), urinary retention requiring discharge with foley catheter ($n = 2$), volume resuscitation for systemic inflammatory response leading to post-operative volume overload requiring diuresis ($n = 1$), penile edema ($n = 1$), gout ($n = 1$), pancreatitis ($n = 2$), delayed operation for gangrenous cholecystitis leading to post-operative deconditioning with inability to walk ($n = 1$), preoperative antibiotics to "cool down" cholecystitis associated with diffuse rash requiring steroids ($n = 1$), hypoxic respiratory failure ($n = 1$), cardiac arrest leading to death ($n = 1$), and multi-organ system failure leading to death ($n = 1$).

Mean ± SD LOS for all patients was 5.0 ± 3.9 d. Patients admitted to the surgical service experienced a shorter LOS compared to patients admitted to the medicine service (Table 2). LOS of 3 d or less was seen more often for surgical patients, while LOS of 6 d or more was observed most often for medicine patients.

Using hierarchical generalized linear models, we evaluated the impact of various factors on time to surgery (Table 3). Adjusting for patient demographics (age and gender), peri-operative co-morbidity level, physical signs, and pre-operative tests, we found that the performance of pre-operative ERCP was associated with increased time to operation. Select physical and laboratory signs (Murphy's sign, temperature > 37.8℃, WBC > 10.2 thousand/UL) had no apparent impact on time to operation in both the unadjusted and adjusted models. Medical admitting team was significantly associated with increased time to operation until ERCP was included in the model (Table 3).

The number of pre-operative diagnostic imaging studies was significantly associated with increased time to operation (Table 3). Time to surgery doubled as the number of diagnostic procedures increased from one to 2 or 3 tests and doubled again when 4 tests were performed. Adjusted models to determine the association between time to surgery and individual studies and combinations of studies revealed that ultrasound alone was associated with the shortest time (mean 23.3, 95% CI 10.7-35.9 h) and the

Table 3 Hierarchical linear models for time to surgery

Model	Estimate mean (h)	Standard error	95% CI	*P* value[4]
ERCP				
Unadjusted				< 0.01
Yes	149	17	115.0-183.0	
No	32.7	4.2	24.3-41.1	
Adjusted for patient demographics[1]				< 0.01
Yes	141.8	17.1	107.7-175.8	
No	33.1	4.1	24.9-41.4	
Adjusted for demographics and co-morbidity				< 0.01
Yes	143.5	16.2	111.1-175.8	
No	33.0	3.9	25.2-40.8	
Adjusted for demographics, co-morbidity and physical signs[2]				< 0.01
Yes	144.7	16.3	112.0-177.4	
No	33.0	3.9	25.1-40.8	
Adjusted for demographics, co-morbidity, physical signs[2] and imaging studies[3]				< 0.01
Yes	134.1	16.2	101.7-166.7	
No	33.6	3.8	26.0-41.2	
Admission service				
Unadjusted				< 0.01
Surgery	30.4	5.1	20.2-40.6	
Medicine	82. 7	11.2	60.3-105.1	
Adjusted for patient demographics				< 0.01
Surgery	31.4	5.1	21.3-41.5	
Medicine	77.6	11.4	54.9-100.4	
Adjusted for demographics and co-morbidity				< 0.01
Surgery	32.1	5.0	22.0-42.1	
Medicine	74.6	11.5	51.7-97.5	
Adjusted for demographics, co-morbidity and physical signs				< 0.01
Surgery	32.2	5.1	21.9-45.5	
Medicine	73.8	11.8	50.1-97.4	
Adjusted for demographics, co-morbidity, physical signs and imaging studies				< 0.05
Surgery	34.2	5	24.2-44.1	
Medicine	64.4	11.9	40.7-88.2	
Adjusted for demographics, co-morbidity, physical signs, imaging studies and ERCP				
Surgery	35.9	4.5	26.8-45.0	
Medicine	55.9	11	33.9-77.9	
Pre-operative diagnostic tests[3]				
Unadjusted				< 0.01
One	26.9	6.4	14.2-39.6	
Two	54.3	8.2	38.0-70.6	
Three	56.7	23.5	9.7-103.7	
Four	125	40.8	43.6-206.4	
Adjusted for patient demographics				< 0.01
One	29.0	6.2	16.6-41.4	
Two	51.5	8.0	35.6-67.4	
Three	48.11	23.6	1.0-95.2	
Four	133.2	39.7	53.8-212.5	
Adjusted for demographics and co-morbidity				< 0.01
One	27.8	6.0	15.8-39.7	
Two	53.6	7.7	38.3-68.9	
Three	46.6	22.6	1.4-91.7	
Four	135.5	38.1	59.4-211.6	
Adjusted for demographics, co-morbidity and physical signs				< 0.01
One	26.9	6.1	14.7-39.1	
Two	55.1	7.9	39.3-70.9	
Three	48.1	23.4	1.4-94.8	
Four	129.3	40.2	48.8-209.8	

[1]Age and sex; [2]Murphy's sign, leukocytosis, and fever; [3]Imaging studies include ultrasound, CT scan, MRCP, and HIDA scan; [4]Measure the differences in means (for more than two means, $P < 0.05$ indicates that at least one pair of means is statistically significantly different).

Table 4 Hierarchical linear models for effect of pre-operative imaging studies on time to surgery

Model	Estimate mean (h)	Standard error	95% CI	*P* value[4]
Unadjusted				< 0.01
All studies done	125.0	36.8	51.4-198.6	
US only	23.6	6.6	10.4-36.8	
US and HIDA	25.2	15.0	0.0-55.2	
CT only	37.0	11.7	13.7-60.3	
CT and MRCP	192.0	36.8	118.4-265.6	
CT and HIDA	59.2	15.0	29.1-89.2	
CT and US	54.9	10.6	33.7-76.2	
US, CT and HIDA	56.7	21.3	14.2-99.2	
Adjusted for patient demographics[1]				< 0.01
All studies done	128.3	36.6	55.1-201.6	
US only	24.7	6.6	11.4-38.0	
US and HIDA	26.2	14.9	0.0-56.0	
CT only	38.3	11.5	15.3-61.3	
CT and MRCP	179.5	37.5	104.5-254.4	
CT and HIDA	58.2	15.0	28.2-88.3	
CT and US	52.4	10.6	31.3-73.5	
US, CT and HIDA	53.9	21.8	10.3-97.4	
Adjusted for demographics and co-morbidity				< 0.01
All studies done	130.6	35.4	59.9-201.4	
US only	23.1	6.5	10.2-36.0	
US and HIDA	29.7	14.4	0.9-58.6	
CT only	39.4	11.1	17.1-61.6	
CT and MRCP	169.6	36.4	96.8-242.5	
CT and HIDA	58.0	14.5	29.0-87.0	
CT and US	55.2	10.3	34.6-75.7	
US, CT and HIDA	52.2	21.0	10.1-94.3	
Adjusted for demographics, co-morbidity and physical signs[2]				< 0.01
All studies done	121.0	36.4	48.1-193.2	
US only	20.4	6.5	7.5-33.3	
US and HIDA	29.7	14.3	1.2-58.3	
CT only	41.3	11.1	19.1-63.6	
CT and MRCP	186.3	36.5	113.1-359.5	
CT and HIDA	60.3	15.2	29.9-90.7	
CT and US	58.4	10.6	37.0-79.7	
US, CT and HIDA	53.5	21.3	10.9-96.0	
Adjusted for demographics, co-morbidity, physical signs and admission service[3]				< 0.01
All studies done	94.7	36.4	21.8-167.5	
US only	23.3	6.3	10.7-35.9	
US and HIDA	33.9	13.7	6.4-61.4	
CT only	42.3	10.6	21.0-63.7	
CT and MRCP	195.4	35.1	125.1-256.8	
CT and HIDA	55.5	14.64	26.2-84.9	
CT and US	52.7	10.42	31.8-73.6	
US, CT and HIDA	49.9	20.36	9.1-90.7	

US: Ultrasound; HIDA: Nuclear Hepatobiliary Scan; MRCP: Magnetic resonance cholangiopancreatography. [1]Age and sex; [2]Murphy's sign, leukocytosis, and fever; [3]Surgical and medical services; [4]Measure the differences in means (for more than two means, $P < 0.05$ indicates that at least one pair of means is statistically significantly different).

combination of CT scan and MRCP with longest time (mean 195.4, 95% CI 125.1-256.8 h) to surgery (Table 4). A trend is present toward decreased risk of complication associated with surgery within 24 h of hospital arrival that becomes non-significant after adjustment for diagnostic imaging studies (Table 5).

DISCUSSION

In this study, we find that 87% of cholecystectomies

Table 5 Hierarchical linear models for association between time to surgery and risk of complication

Model	Odds ratio	95% CI	*P* value
Unadjusted (h)			> 0.01
< 24	0.03	0.004-0.24	
24-48	0.3	0.05-1.52	> 0.05
48-72	0.2	0.02-1.74	> 0.05
> 72 (reference)	1		> 0.05
Adjusted for patients demographics (h)[1]			
< 24	0.05	0.003-0.65	
24-48	0.38	0.04-4.03	
48-72	0.27	0.01-5.01	
> 72	1		
Adjusted for demographics and co-morbidity (h)			
< 24	0.05	0.003-0.74	
24-48	0.44	0.04-5.00	
48-72	0.26	0.01-4.91	
> 72 (reference)	1		
Adjusted for demographics, co-morbidity and physical signs (h)[2]			
< 24	0.04	0.003-0.45	
24-48	0.33	0.03-3.49	
48-72	0.25	0.01-5.06	
> 72	1		
Adjusted for demographics, co-morbidity, physical signs and imaging studies (h)[3]			
< 24	0.08	0.004-1.61	
24-48	0.66	0.04-12.2	
48-72	0.41	0.01-13.5	
> 72	1		

[1]Age and sex; [2]Murphy's sign, leukocytosis, and fever; [3]Imaging studies included abdominal ultrasound, CT scan, MRCP, and HIDA scan. Abbreviations as in Table 4.

occurred within 72 h of arrival at the hospital and half of them within 24 h, indicating an overall timely approach. Several studies have shown that performing LC within 72 h of presentation does not increase morbidity and mortality[6,9]. Early LC, furthermore, has been shown to have a lower complication rate, decreased length of stay, decreased cost, and a shorter post-operative recovery period compared to delayed LC[5,7,8,16,17]. The results from a meta-analysis by Papi *et al* of 12 prospective randomized trials from 1970-2000 (9 addressing open cholecystectomy, 3 addressing LC), showed that there was a decrease in conversion to open procedure when LC was done early (17.6% *vs* 25.7%) and there were fewer post-operative complications (10.9% *vs* 15.6%)[17]. Another benefit of early surgery proved to be a shorter length of hospital stay (6.3 d *vs* 9.9 d).

Our analysis suggests a number of potential ways to speed up time to cholecystectomy. The number of pre-operative procedures was significantly associated with adjusted time to surgery, especially when four or more tests were done. Performance of MRCP and CT scan had by far the longest associated adjusted time to surgery. While the need for ERCP and MRCP will always result in some delay in surgery, these studies are routinely available within 24 h at our institution, often on the same day as requested. Efforts to streamline the decision process leading to ERCP and MRCP and then providing these studies promptly when requested may lead to earlier surgery for patients with acute cholecystitis. Additionally, more judicious use of pre-operative diagnostic imaging studies would also reduce delay. Although the sample size was small, not surprisingly, the performance of ERCP was also associated with delayed time to operation.

Also noted was that patients admitted to the medical service experienced over twice the wait for surgery compared to those going to the surgical service. No doubt some of this delay represents treatment of co-morbid illnesses, which were significantly more common in patients admitted to the medical service. However, patients on the medical service also had more imaging procedures performed, mostly abdominal CT scans, indicating confusion over the diagnosis and contributing to delays in definitive surgical therapy. Surgeons directly caring for patients with abdominal pain may be more apt to make the early diagnosis of acute cholecystitis with fewer imaging studies than medical physicians and, thereby, bring those patients to operation earlier. Although admitting patients with abdominal pain suspicious for cholecystitis to the surgical service may reduce time to cholecystectomy, it must be weighed against the advantages of having patients with active co-morbidities managed on the medical service with surgical consultation.

Our findings raise the question whether the benefit seen by early surgery is conferred mainly on those patients operated on within 24 h of arrival. A significant decreased risk of complications was seen in this subset of patients, except in the model including adjustment for diagnostic imaging studies. A reasonable inference is that this subset of patients may be so straight forward that little diagnostic uncertainty exists, fewer tests are required, and admission to the surgical service is favored. Conversely, those patients about whom diagnostic uncertainty is present or who are chosen for "cooling off" with pre-operative antibiotics may be placed at greater risk of complications because of the prolonged inflammatory state. Acute inflammation creating edema in the gallbladder submucosa can mature into a chronic inflammatory response with neovascularization and fibrosis, which can make a delayed procedure potentially more difficult[18]. Often, an operation will be deferred, especially in patients with pre-existing co-morbidities, in an attempt to maximize a patient's medical tolerance of an operative insult. Firm evidence is lacking for whether this delayed approach is truly the best. Indeed, a recent meta-analysis comparing LC within 7 d *versus* longer than 6 wk after index admission showed no clear benefit from waiting[19]. Although the complications observed in the patients in this analysis can be directly or indirectly linked to acute cholecystitis and surgery, no causal connection between them and any delay in operating can be drawn. A prospective trial is likely required to determine the optimal times for LC.

Somewhat surprising was that what many would deem a classic clinical presentation with fever, leukocytosis, and Murphy's sign was not associated with time to surgery. In a retrospective study of patients undergoing nuclear hepatobiliary scans by Singer *et al*[20], fever had a positive predictive value of 100% and a sensitivity of 14.6% for a positive scan. A positive Murphy's sign had 97.2% sensitivity and 93.3% positive predictive value. In their

systematic review of presenting signs and symptoms that may warrant investigation for cholecystitis, Trowbridge *et al*[21] found that no clinical or laboratory finding had a likelihood ratio (LR) sufficient to rule in or out cholecystitis. Murphy's sign, however, appeared the most useful, with a positive LR of 2.8, 95% CI 0.8-8.6. In our study, possibly the variable skill of the clinicians assessing for cholecystitis and the ready availability of imaging studies mitigated any diagnostic benefit from the presence of these findings that would have sped up care.

We chose in this study to use the AHA/ACC risk classification scheme rather than the American Society of Anesthesiologists-Physical Status (ASA-PS) score for risk stratification. While the ASA-PS score's simplicity and widespread use makes it an attractive index of risk, it is subjective and not explicitly intended for estimation of operative risk[22]. Furthermore, we had no measure of inter-anesthesiologist agreement on ASA-PS score and numerous anesthesiologists, so we could not control for variations at the level of the grader. While the AHA/ACC guidelines were derived for cardiovascular risk, it contains a detailed list of factors plausibly conferring risk for non-cardiac complications[23-25].

In summary, our analysis indicates good adherence to the desirable practice of performing cholecystectomies within 3 d of presentation to the hospital and raises the question of whether even prompter surgery may reduce the risk of complications. Efforts to expedite cholecystectomy should consider factors that may contribute to delay in surgery such as ERCP and MRCP, the combination of CT scan and MRCP, and admission to a medical service.

COMMENTS

Background

The optimal timing of surgery for acute cholecystitis (AC) has not been precisely established. We reviewed 70 consecutive cases of AC, confirmed by pathological examination, and identified several factors which led to delay in surgery and which resulted in increased complications because of that delay.

Research frontiers

We used a hierarchical statistical analysis model to identify the relevant factors leading to surgical delay.

Innovations and breakthroughs

While other authors have reviewed the timing of surgery for AC, none have identified specific causes resulting in delay nor reviewed the consequences of delaying surgery.

Applications

We found patients with AC who are admitted to the Medical Service, because of medical co-morbidities, experience significant delays in definitive surgical treatment and have higher post-operative complication rates, even when corrected for the existing co-morbidities. The delay results primarily from an increased number of radiologic imaging studies performed on patients admitted to the Medical Service. Patients with medical co-morbidities in addition to AC require prompt stabilization of their underlying medical illnesses and definitive surgery within 48 to 72 h following admission.

Terminology

The hierarchical statistical analysis used in this article allows multiple variables to be compared, identifying those that impact surgical timing and eliminating individual practice patterns from affecting the analysis.

Peer review

This study by Daniak *et al* is based on a retrospective evaluation of records from patients who underwent cholecystectomy. The authors compared the time of hospital staying between patients admitted in surgery and medical service. Interestingly, they found that admission to the medical service was associated to longer time to surgery compared to admission to surgery service. The study is original, well designed and performed, and results are relevant.

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RAPID COMMUNICATION

An easier method for performing a pancreaticojejunostomy for the soft pancreas using a fast-absorbable suture

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Abstract

AIM: To clarify the usefulness of a new method for performing a pancreaticojejunostomy by using a fast-absorbable suture material irradiated polyglactin 910, and a temporary stent tube for a narrow pancreatic duct with a soft pancreatic texture.

METHODS: Among 63 consecutive patients with soft pancreas undergoing a pancreaticoduodenectomy from 2003 to 2006, 35 patients were treated with a new reconstructive method. Briefly, after the pancreatic transaction, a stent tube was inserted into the lumen of the pancreatic duct and ligated with it by a fast-absorbable suture. Another tip of the stent tube was introduced into the intestinal lumen at the jejunal limb, where a purse-string suture was made by another fast-absorbable suture to roughly fix the tube. The pancreaticojejunostomy was completed by ligating two fast-absorbable sutures to approximate the ductal end and the jejunal mucosa, and by adding a rough anastomosis between the pancreatic parenchyma and the seromuscular layer of the jejunum. The initial surgical results with this method were retrospectively compared with those of the 28 patients treated with conventional duct-to-mucosa anastomosis.

RESULTS: The incidences of postoperative morbidity including pancreatic fistula were comparable between the two groups (new; 3%-17% *vs* conventional; 7%-14% according to the definitions). There was no mortality and re-admission. Late complications were also rarely seen.

CONCLUSION: A pancreaticojejunostomy using an irradiated polyglactin 910 suture material and a temporary stent is easy to perform and is feasible even in cases with a narrow pancreatic duct and a normal soft pancreas.

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Key words: Pancreaticojejunostomy; Duct-to-mucosa contact method; Fast-absorbable suture; Irradiated polyglactin 910; Pancreatic fistula

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INTRODUCTION

Leakage of the pancreaticoenterostomy remains a major cause of postoperative morbidity and mortality after pancreaticoduodenectomy[1-7]. Many factors have been identified that are associated with an increased incidence of this complication. Among them, a small pancreatic duct size with a soft pancreas creates one of the technical hurdles to the completion of the anastomosis, and is known to be a risk factor for major leakage[3,8-13]. Several methods have been advocated to reduce the occurrence of leakage[5,14-21], but the best technique is still a subject of debate[22-29].

We established a new anastomotic technique for pancreaticojejunostomy by using a fast-absorbable suture material, irradiated polyglactin 910, and a temporary stent tube.

MATERIALS AND METHODS

One hundred and one consecutive patients underwent an elective pancreaticoduodenectomy for hepato-biliary and pancreatic disease between February 2003 and January 2007 at the Hirosaki University Hospital. Among them, 63 patients, in whom the pancreas was macroscopically normal and soft in texture, and in whom a pathological examination of the pancreatic cut end confirmed the absence of fibrosis or pancreatitis, were included in

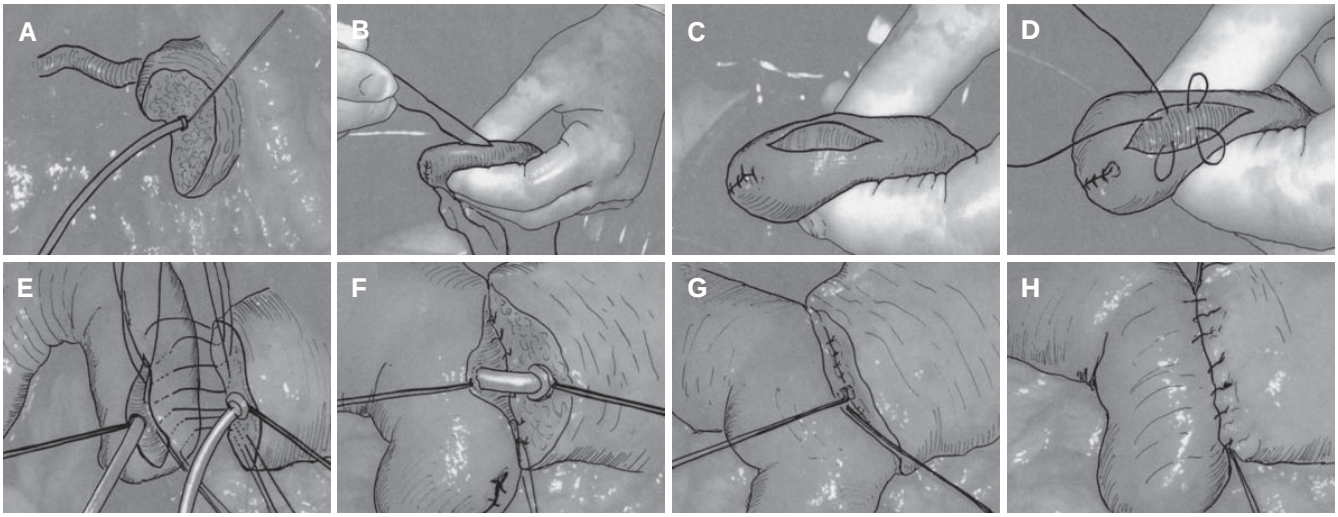


Figure 1 New Duct-to-mucosa contact method. **A**: The stent tube was ligated with the pancreatic duct by an irradiated polyglactin 910 suture; **B**, **C**, **D**: The seromuscular layer of the jejunal wall was cut. The submucosa was exposed and a purse-string suture using an irradiated polyglactin 910 suture was performed at the center, where another tip of the stent tube was introduced and ligated by this suture material; **E**, **F**, **G**: After the posterior side of the pancreatic parenchyma was anastomosed to the seromuscular layer of the jejunal wall, two stay sutures of the stent tube were ligated to approximate the pancreatic ductal cut end and the jejunal mucosa; **H**: The pancreaticojejunostomy was completed by adding an anterior suture to the pancreatic parenchyma and the jejunal wall.

this study. The reconstruction of the pancreatic stump after the pancreaticoduodenectomy was performed by a pancreaticojejunostomy in two fashions, the conventional duct-to-mucosa anastomosis, or a new duct-to-mucosa contact method. Five surgeons performed either of the anastomotic techniques, as was their preference, however, one of the authors, KH, supervised all operations and the procedures were strictly standardized in the institute.

The postoperative management was prescribed according to the clinical pathway. The amylase level in the peripancreatic external drain was measured on postoperative d 1, 3 and 6. In this study, a pancreatic fistula was defined by four internationally well-documented criteria, which are described as follows: (1) drainage of more than 50 mL per day of fluid with an amylase content above three times the serum amylase activity on and after postoperative d 10 as described by Yeo *et al*[30] (Definition A); (2) drainage of more than 50 mL per day of fluid with an amylase content above three times the serum amylase activity on and after postoperative d 7 as described by Park *et al*[31] (Definition B); (3) drainage of more than 10 mL per day of fluid with an amylase content above three times the serum amylase activity on and after postoperative d 4 as described by Buchler *et al*[32] and Pederzoli *et al*[33] (Definition C); (4) drainage of any measurable volume of fluid with an amylase content greater than three times the normal serum value on or after postoperative d 3 according to the International Study Group of Pancreatic Fistula (ISGPF) classification scheme[34,35]. According to the ISGPF definition, outcomes were divided into four grades: (1) no fistula; (2) Grade A: biochemical fistula without clinical sequelae; (3) Grade B: fistula that shows clinical symptoms or requires any therapeutic intervention; (4) Grade C: fistula with severe clinical sequelae. The occurrence of Grade B or C fistulas is included in the postoperative morbidity. A delayed gastric emptying is defined as the need for nasogastric drainage for more than 10 d[2,7,12], or as the delay of the resumption of a regular diet beyond 14 d postoperatively[36]. Computed tomography (CT), either a helical CT or a multidirector row CT, was performed serially before discharge and on the third and sixth postoperative month to rule out cancer recurrence. Based on these findings, the diameter of the main pancreatic duct ventral to the base of the superior mesenteric artery was measured by an enhanced computed tomogram. A progression of pancreatic ductal dilatation was defined by the diameter on the sixth month CT being 130% or greater than that before the hospital discharge. The glucose tolerance was evaluated serially by blood hemoglobin A_1C or a 75-g oral glucose tolerance test at the same intervals as the CT scans. Diabetes mellitus was defined as a fasting glucose level greater than 1.26 g/L or a two-hour glucose level greater than 2 g/L.

The data were collected prospectively for all patients and included the preoperative historical information and laboratory data, the details of the operative procedure, the pathology, and the clinical information regarding the postoperative course, postoperative morbidity and mortality, and the late complications occurring at least 6 mo postoperatively.

The statistical analyses were performed using the SPSS 11.0 statistical software. The comparisons between the two groups were carried out by Chi-square test for the categorical data and Student's *t*-test for the continuous data. The continuous variables were reported as the mean ± SD. *P* values less than 0.05 were thought to be significantly different.

Surgical procedures

New Duct-to-mucosa contact method: The pancreatic transection was performed with an electrocautery or a scalpel. A stent tube (5, 6, or 7.5 Fr, Sumitomo Bakelite, Tokyo, Japan) with a notch near the tip was inserted into the lumen of the main pancreatic duct. The stent tube was ligated with the pancreatic duct near the cut end by a 4-0 fast-absorbable synthetic suture, irradiated polyglactin 910 (vicryl RAPIDE™, Ethicon, Somerville, N.J.) (Figure 1 A),

Table 1 Preoperative and operative data

	New method *n* (%)	Conventional method *n* (%)	*P*
Patient number	35	28	
Male/female	20/15	11/17	0.77
Age (yr)	64.1 ± 9.2	68.0 ± 8.6	0.09
Preoperative biliary drainage	28 (80)	19 (68)	0.27
Diabetes mellitus	8 (23)	13 (46)	0.05
Weight loss > 10%	4 (11)	4 (14)	0.74
Operative indications			< 0.01
Biliary adenocarcinoma[1]	28 (80)	10 (36)	
Ampullary adenocarcinoma	2 (6)	2 (7)	
Duodenal adenocarcinoma	1 (3)	1 (4)	
Pancreatic adenocarcinoma	0	12 (43)	
Other pancreatic neoplasm[2]	4 (11)	3 (11)	
Type of resection			0.44
Classical Whipple	6 (17)	7 (25)	
Pylorus-preserving	29 (83)	21 (75)	
Pancreaticojejunostomy	35 (100)	28 (100)	-
Diameter of pancreatic duct (mm)	2.4 ± 0.5	4.2 ± 1.9	< 0.01
Operative time (min)	392 ± 72	418 ± 110	0.24
Time after resection (min)	187 ± 56	181 ± 66	0.74
Blood loss (g)	1074 ± 490	1112 ± 607	0.78
Patients transfused	5 (14)	5 (18)	0.7

[1]Carcinomas of the bile duct and the gallbladder; [2]Non-invasive intraductal papillary-mucinous neoplasm, islet cell tumor, and lymphangiohemangioma of the pancreatic head.

The notch functions to prevent the tube from dislodging. The jejunal limb was moved to the pancreatic cut end by a retro-mesenteric route. The seromuscular layer of the jejunum was cut at the anti-mesenteric side, and the serosal side of the mucosa was exposed by the same width as the pancreatic cut surface (Figure 1B and C). A purse-string suture was made by using a 4-0 irradiated polyglactin 910 at the center of the mucosa, where another tip of the stent tube was introduced into the intestinal lumen, and ligated by this fast-absorbable suture (Figure 1D). After the posterior side of the pancreatic parenchyma was anastomosed to the seromuscular layer of the jejunal wall by using 4-0 polydioxanone suture, a long-lasting absorbable synthetic suture (PDS-Ⅱ™, Ethicon) (Figure 1E and F), two stay sutures of irradiated polyglactin 910 were ligated together to approximate the ductal cut end and the jejunal mucosa (Figure 1G). No other suture was added to the duct-to-mucosa contact. Next, the anterior side of the pancreatic parenchyma was anastomosed to the seromuscular layer of the anterior wall of the jejunum by polydioxanone sutures (Figure 1H). The other tip of the tube was introduced extracorporeally through the transhepatic or transintestinal route. The pancreatic juice flowed through the lumen of the stent tube and then drained externally. The whole anastomotic procedure usually required 10 to 15 min at maximum.

After confirmation of a stable anastomosis by the tube pancreatography on postoperative d 14, the tube could be pulled out without any resistance.

Conventional Duct-to-mucosa anastomosis

After the posterior side of the pancreatic parenchyma was anastomosed to the wall of the seromuscular layer of the jejunum by using 4-0 polydioxanone sutures, the pancreatic ductal end was anastomosed to the small hole at the antimesenteric side of the jejunal limb by using 5-0 polydioxanone sutures in an interrupted fashion. Then the anterior side of the pancreatic parenchyma was anastomosed to the wall of the seromuscular layer of the jejunum by using 5-0 polydioxanone sutures. No stent tube was used.

RESULTS

The new duct-to-mucosa contact method was applied to 35 patients, while the conventional duct-to-mucosa anastomosis was performed in 28 patients (Table 1). The male-to-female ratio was the same between the two groups; however the duct-to-mucosa contact method was applied to older patients. There were no differences in the presence of jaundice, diabetes mellitus, or malnutritional status, which was defined as a 10% or greater weight loss.

The operative indications were different between the two groups. The duct-to-mucosa contact method was applied more frequently to biliary cancer, while the conventional duct-to-mucosa anastomosis was preferred in pancreatic cancer. The diameters of the pancreatic duct at the cut surface were significantly smaller in the duct-to-mucosa contact method group (2.4 ± 0.5 *vs* 4.2 ± 1.9 mm, $P < 0.01$). The total operative time, as well as the time after resection, was the same between the two groups. The amount of blood loss and the number of patients transfused were comparable in both groups.

Postoperative complications occurred equally to both groups (Table 2). Pancreatic fistula was noted in 3% of the patients in the duct-to-mucosa contact method group and in 7% in the duct-to-mucosa anastomosis group ($P = 0.43$) according to Definition A. By the more strict definitions of Definition B and C, pancreatic fistula was found in 8% and 17% by the new method, while in 8% and 11% by the conventional method, respectively ($P = 0.77$ and $P = 0.47$). Fistulas of any grade (Grade A-C) by the ISGPF definition occurred in the same degree. Moreover, the incidences of Grade B or C fistulas, which were categorized into clinically relevant fistulas, were also comparable between the two groups (17% *vs* 14%, $P = 0.76$). Intestinal bleeding from the ruptured pseudoaneurysm of the common hepatic artery, which occurred in a patient treated with duct-to-mucosa anastomosis on postoperative d 21, was successfully treated with radiological intervention using a self-expandable metallic stent. Other abdominal and non-abdominal complications, both infectious and non-infectious, were comparable between the two groups. No operative mortality was found in this series. The length of hospital stay and the re-admission rate were equal in both groups.

A late pancreatic ductal dilatation, as determined by a comparison with the ductal diameter before discharge, was noted in one patient in each group. An exacerbation of diabetes mellitus was rarely found in either group, and the recovery of body weight was comparable between the two groups.

DISCUSSION

Pancreatic fistula is the major cause of postoperative

Table 2 Postoperative course, complications and late outcome

	New method *n* (%)	Conventional method *n* (%)	*P*
Morbidity[1]	16 (46)	11 (39)	0.61
Abdominal complications	15 (43)	10 (36)	0.56
Pancreatic fistula			
Definition A (Yeo *et al*)	1 (3)	2 (7)	0.43
Definition B (Park *et al*)	3 (8)	3 (8)	0.77
Definition C (Buchler *et al*)	6 (17)	3 (11)	0.47
Grade B or C by ISGPF definition	6 (17)	4 (14)	0.76
ISGPF definition			
No fistula	20 (57)	21 (75)	0.23
Grade A	9 (26)	3 (11)	
Grade B	6 (17)	3 (11)	
Grade C	0	1 (4)	
Biliary leakage	0	0	-
Intraabdominal abscess	2 (6)	4 (14)	0.25
Intraabdominal bleeding	0	0	-
Intestinal bleeding	0	1 (4)	0.26
Delayed gastric emptying	2 (6)	0	0.20
Liver abscess	0	1 (4)	0.26
Wound infection	2 (6)	0	0.20
Other complications	1 (3)	2 (7)	0.43
Pneumonia	0	1 (4)	0.26
Pulmonary embolism	0	1 (4)	0.26
Catheter fever	1 (3)	0	0.37
Re-operation	0	0	-
In-hospital mortality	0	0	-
Hospital stay (d)	29.5 ± 10.9	28.0 ± 12.2	0.60
Re-admission within 30 d	1 (3)	1 (4)	0.87
Late complications[2]			
Progression of ductal dilatation	1 (3)	1 (4)	0.87
Exacerbation of diabetes	1 (3)	1 (4)	0.87
Body weight gain (%)[3]	100.3 ± 4.6	101.3 ± 9.0	0.65

[1]Total patients with one or more complications; [2]Comparison before hospital discharge and on the sixth month; [3]Percent body weight gain at 6th month in comparison to that at the time of discharge.

morbidity and mortality after pancreaticoduodenectomy. The incidence is estimated to be 5% to 30%[1,5-7,34,35], which varies according to the definition[34,35]. Many risk factors for pancreatic fistula have been disclosed, among which the most significant factors are thought to be a soft pancreatic texture, a narrow pancreatic duct, and a high output of pancreatic juice[3,8-13].

Pancreaticoenterostomy is the most physiologic method to deal with the remnant pancreatic stump, because the continuity of the pancreatic exocrine and intestinal functions can be restored[37-39]. Indeed, a pancreaticoenterostomy, either *via* a pancreatico-jejunostomy or a pancreaticogastrostomy, is easy to perform in cases of a dilated pancreatic duct with a firm texture of the pancreatic parenchyma, but in cases with a normal pancreas, it is quite hard to anastomose a narrow pancreatic duct and a fragile parenchyma to the jejunal or gastric wall[11,13,40]. To overcome the technical difficulties of performing a pancreaticoenterostomy of a soft pancreas, we have developed a new anastomotic technique of a "duct-to-mucosa contact method" by using a fast absorbable suture material and a temporary stent tube.

In this method, the pancreatic ductal end is approximated to the serosal side of the jejunal mucosa by ligating two stay sutures of the temporary stent tube. This method can be applied easily to a narrow pancreatic duct if the stent tube can be inserted into it. In our series, the mean diameter of the pancreatic duct in the duct-to-mucosa contact method group was significantly smaller than that in the conventional duct-to-mucosa anastomosis group. The surgeons tended to choose this method for the narrow duct because it was a very simple procedure and they did not need to hold a fine needle or use a surgical microscope[21].

The priority of this method is the use of an irradiated polyglactin 910 suture. This synthetic absorbable suture material has an initial strength comparable to that of nylon but it loses its tensile strength rapidly, being 50% at 5 d and almost none at 14 d, according to the commercial information[41]. An added advantage of this material is the very mild inflammatory reaction of the surrounding tissues[42,43]. These characteristics favor its use in the field of oral surgery[44,45] as well as in pediatric[42], gynecological[46,47], and plastic surgery[48,49].

In this duct-to-mucosa contact method, the irradiated polyglactin 910 suture was used to secure the complete external drainage of the pancreatic juice and to approximate the pancreatic duct and the mucosa for about a week. The anastomosis of the pancreatic parenchyma and the intestinal seromuscular wall was secured by the more long-lasting absorbable synthetic sutures comprised of polydioxanone[50]. In most cases, the color of the drained pancreatic juice changes from transparent to bile-staining by POD 14, when the irradiated polyglactin 910 has already been degraded and the stent can be removed without any resistance. These phenomena suggested to us that the exposure of the pancreatic juice to the anastomotic site could be avoided for about 2 wk postoperatively.

It has been suggested that the rate of formation of a pancreatic fistula varies according to the definition. Bassi *et al*[34] summarized the current pancreatic fistula concept by analyzing 26 definitions described in the past 10 year, and applied four definitions to a group of 242 patients undergoing a pancreaticojejunostomy after a pancreatic resection. They found the incidence of pancreatic fistula to range from 9.9% to 28.5% according to the different definitions applied. Pratt *et al*[35] also reported incidences of pancreatic fistula between 9.1% and 19.3% by applying different definitions to 176 patients undergoing a pancreaticoduodenectomy. As a result, it is quite important to use a universally accepted definition of pancreatic fistula to compare the different aspects of the new surgical method with the conventional one. In this study, we applied three well-documented definitions as described by Yeo *et al* (1995)[7], Park (1998)[31], and Buchler (1992)[32] and Pederzoli (1994)[33] to help compare our results with those of previous reports. We examined a high risk group of pancreatic fistula in which the pancreatic texture was soft and the pathology confirmed the absence of fibrosis and pancreatitis change. Nevertheless, the incidences were comparable to the overall incidences of the previous reports using these definitions[1,3,6,7,12,13,22,29], or were even lower than those in cases restricted to the soft pancreas[9,10]. In a current objective definition by the ISGPF classification scheme, Pratt *et al*[35] reported an overall incidence of 11.9% in Grade B fistula and 3.4% in

Grade C fistula, which were comparable with our results of 14.4% in Grade B fistula and 1.6% in Grade C fistula. A subgroup analysis of the incidences of pancreatic fistula limited to the soft pancreas in each Grade according to the ISGPF definition has not yet been reported to date.

Consequently, the total incidence of the postoperative infectious and non-infectious complications was not different from that with conventional duct-to-mucosa anastomosis. The late status of the anastomotic site also seemed to be stable according to the serial measurements of the pancreatic ductal diameter as determined by CT scans. Good recovery of the nutritional status and avoiding worsening of the diabetic symptoms seemed to be primarily due to the uniform preoperative backgrounds of the normally functioning pancreas in this series, but these results may indirectly favor the use of this new method.

The primary aim of this study was to introduce a new method of performing a pancreaticojejunostomy, because this method is quite easy to perform even in a difficult anastomosis of a narrow duct of a normal soft pancreas. Indeed any conclusion for the surgical method needs to await randomization or a good cohort study, but our preliminary results were comparable with those previously reported from mass volume centers. These facts seem to justify the carrying out of a randomized prospective study to compare this new method with appropriate controls.

COMMENTS

Background

A narrow pancreatic duct with a soft pancreatic texture creates a high risk for the development of a pancreatic fistula after pancreaticoduodenectomy. Several methods have been advocated to reduce the occurrence of leakage, but the best technique is still a subject of debate.

Research frontiers

The authors developed a new method for performing a pancreaticojejunostomy with a fast-absorbable suture material, irradiated polyglactin 910, and a temporary stent tube.

Innovation and breakthrough

This easy-to-perform method was feasible in cases with narrow pancreatic duct and a soft pancreas. Both early and late surgical results were comparable to or better than the methods ever reported.

Applications

This method is expected to give a new idea of overcoming the difficulties of pancreaticoenterostomy for a soft pancreatic texture with a narrow pancreatic duct.

Peer review

In this study, 35 consecutive patients with a soft pancreas undergoing a pancreaticoduodenectomy from 2003 to 2006 were treated by a new reconstructive method. This work adds significant information for future development of pancreaticojejunostomy therapy.

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RAPID COMMUNICATION

Endoscopic transpapillary brush cytology and forceps biopsy in patients with hilar cholangiocarcinoma

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Abstract

AIM: To evaluate the sensitivity of brush cytology and forceps biopsy in a homogeneous patient group with hilar cholangiocarcinoma.

METHODS: Brush cytology and forceps biopsy were routinely performed in patients with suspected malignant biliary strictures. Fifty-eight consecutive patients undergoing endoscopic retrograde cholangiopancreatography (ERCP) including forceps biopsy and brush cytology in patients with hilar cholangiocarcinoma between 1995-2005.

RESULTS: Positive results for malignancy were obtained in 24/58 patients (41.4%) by brush cytology and in 31/58 patients (53.4%) by forceps biopsy. The combination of both techniques brush cytology and forceps biopsy resulted only in a minor increase in diagnostic sensitivity to 60.3% (35/58 patients). In 20/58 patients (34.5%), diagnosis were obtained by both positive cytology and positive histology, in 11/58 (19%) by positive histology (negative cytology) and only 4/58 patients (6.9%) were confirmed by positive cytology (negative histology).

CONCLUSION: Brush cytology and forceps biopsy have only limited sensitivity for the diagnosis of malignant hilar tumors. In our eyes, additional diagnostic techniques should be evaluated and should become routine in patients with negative cytological and histological findings.

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Key words: Brush cytology; Forceps biopsy; Cholangiocarcinoma; Klatskin tumor; Endoscopy; Diagnostic approaches

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INTRODUCTION

Current epidemiological data analysis reveals an increasing incidence and mortality from cholangiocarcinoma[1-4]. Cholangiocarcinomas are topographically categorized as intrahepatic or extrahepatic carcinomas. Extrahepatic cholangiocarcinomas are further subdivided into hilar, medial and distal carcinomas. The most common type are hilar cholangiocarcinomas which are classified into 4 stages according to the bismuth classification: stage I or II for tumors expanding up the hilus, type III A/B infiltrating right or left hepatic duct, and stage IV with infiltration of both hepatic ducts and sub segments[5]. Because of late presentation of symptoms and the difficult distinction between benign and malignant strictures the prognosis of patients with hilar cholangiocarcinoma is poor, and survival data for advanced stages reported so far show a very limited life expectancy[6-8]. Surgery is the only curative treatment in patients with cholangiocarcinoma. The results are more favourable for patients with early-stage disease. Therefore, a reliable diagnostic procedure is of great importance for patient's chance for survival. Cholangiocarcinomas are diagnosed by a combination of imaging techniques and endoscopic procedures, including endoscopic retrograde cholangiography and/or percutaneous transhepatic cholangiography[9-13]. Cholangiocarcinomas often grow longitudinally along the bile duct rather than in radial direction

away from the bile duct. Consequently, imaging techniques including ultrasound, computed tomigraphy (CT), and magnetic resonance imaging (MRI) are of limited sensitivity for the detection of the malignant lesion[14-17].

Biliary tissue collection during endoscopic procedures is widely used for distinction between benign and malignant strictures and provides the only definitive diagnosis that can be used for establishing therapeutic strategies. To obtain tissue samples, brush cytology and/or forceps biopsy were routinely performed in patients with suspected malignant biliary strictures. Several studies have evaluated the cancer detection rate of brush cytology since the first description by Osnes *et al*[18] in 1975. Brush cytology is the most common tissue sampling technique and can be performed for most biliary strictures detected by endoscopic retrograde cholangiography. It is generally safe, requires little time, and is technically easier compared to forceps biopsy. The sensitivity of brush cytology for diagnosis of malignant biliary strictures ranges from 30% to 60% in most published series[19-21]. Tissue samples for histological investigation can be obtained from biliary strictures by using forceps. This technique is more time consuming than brushing and is less widely used, but it provides a sample of subepithelial stroma. Consequently, only histological investigation allows diagnosing invasive growth. In patients with malignant biliary stricture the overall cancer detection rate of forceps biopsy is higher compared to brush cytology, ranging form 43% to 81%[22-24].

To date, no study exists investigating the sensitivity of these techniques in patients with hilar cholangiocarcinoma (Klatskin tumors). The present study was designed to address the question of diagnostic sensitivity for endoscopic transpapillary brush cytology and forceps biopsy in a homogeneous group of 58 patients with hilar cholangiocarcinoma.

MATERIALS AND METHODS

The study included 58 consecutive patients (31 male, 27 female, median age 68 ± 10.6 years) with malignant hilar bile duct tumors who were treated from 1995 to 2005 in the Ⅱ. Medizinische Klinik, Klinikum rechts der Isar at the Technical University Munich. The biliary stricture localization was classified in relation to the confluence of the hepatic ducts as described by bismuth[5]. Bismuth stage was classified by endoscopic retrograde cholangiography at the time of primary diagnosis.

Patients included into this study were identified by using an endoscopic database. The analysis of our database was performed as follows: First, all patients with bile duct strictures diagnosed in the period from 1995 to 2005 were identified. Subsequently, cholangiograms of these patients were reviewed and the bile ducts strictures were divided according to its localization. In the next step we tried to clarify the genesis of the hilar stricture by analysing patient records including follow up. In total, 96 patients with hilar cholangiocarcinoma could be identified. In 58 out of 96 patients forceps biopsy and brush cytology was performed.

All of the following inclusion criteria had to be confirmed: (1) Diagnosis of hilar cholangiocarcinoma (histologically/cytologically positive for malignancy or patients for whom the subsequent clinical course confirmed malignancy); (2) Transpapillary brush cytology and forceps biopsy as diagnostic approach. Exclusion criteria were as following: (1) Intrahepatic cholangiocarcinoma; (2) Distal cholangiocarcinoma; (3) Biliary obstruction due to liver metastasis; (4) Hepatocellular carcinoma; (5) No tumor progression in patients with negative histology/cytology; (6) Survival time more than 18 mo in patients with negative histological/cytological investigation. The exclusion criteria were scheduled in order to achieve a homogeneous patients group with hilar cholangiocarcinoma without tumors of different origin.

In all patients, routine diagnostic procedures included abdominal ultrasound, abdominal CT scans and endoscopic retrograde cholangiopancreatography (ERCP) with tissue sampling. After reception of the histology results, patients with strictures were associated with tumors located in the hilar region, respectability was primarily discussed with the abdominal surgeon. In 28 selected patients cholangioscopy was performed. Overall 16 out of 58 patients were transferred to the surgery unit and operation was performed. In patients with negative histology/cytology after first attempt of forceps biopsy/brush cytology another diagnostic approaches including percutaneous ultrasound guided fine needle biopsy, endoscopic ultrasound guided fine needle biopsy, or percutaneous transhepatic cholangioscopic guided biopsy was performed. In patients with furthermore unclear diagnosis, another attempt of transpapillary tissue sampling was performed after 3 mo.

Endoscopic retrograde cholangiography (ERC) was performed with a standard videoduodenoscope Olympus TFJ 160-R (Olympus, Hamburg, Germany). The first ERC comprised an endoscopic sphincterotomy (EPT) which was performed using an Olympus papillotome (Olympus, Hamburg, Germany) introduced over a Terumo guide wire. Under radiographic guidance using contrast fluid bile duct strictures were localized. The forceps was advanced as far as possible into the stricture in the closest position, then forceps was opened and the specimen was obtained. If the forceps could not enter the stricture, the open forceps was gently pushed against the distal end of the bile duct stricture. The specimen was obtained by closing the forceps in this position and fixed in 4% formalin. Bile duct strictures were brushed with multiple, rapid, to and fro movements using a standard 6 French (F) Geenen spring tip sheathed cytology brush inserted over a standard guide wire. The cellular material adherent to the brush was directly transferred to a glass slide in the endoscopy room. The brush was subsequently stored in 50% ethanol and processed at the Institute of Pathology to collect the remaining material. The specimens were evaluated by an experienced cytopathologist. Cytological results were recorded as positive or negative for malignant cells. According to the usual cytologic and histologic classification system, in the current study suspicious and positive specimens were considered as positive, whereas negative specimens and unspecific reactive changes were classified as negative.

In two patients, passage of the Y-shaped stenosis of

Table 1 Patients characteristics, physical and laboratory parameters on admission

	Patient characteristics	Standart values	Scale unit
Number of patients	58	-	-
Gender	Male (m): 31 (53.4%)	-	-
	Female (f): 27 (46.6%)	-	-
Bismuth Ⅱ	7	-	-
Bismuth Ⅲ	25	-	-
Bismuth Ⅳ	26	-	-
Bilirubin	6.9 ± 7.6	< 1.2	mg/dL
Alkaline phosphatase	559 ± 270	40-120	U/L
γ-GT	404 ± 362	< 66	U/L
Leucocytes	8.8 ± 3.8	4-9	G/L

Table 2 Number of tissue sampling sessions

Number of tissue sampling sessions	Number of patients	Number of samples
1 session	47	47
2 sessions	8	16
3 sessions	2	6
4 sessions	1	4
	58	73

a complex hilar tumor did not allow a stable passage of a wire guided brush into the periphery after dilatation or use of an internal bougie. Since the tip of the brush runs parallel to the wire although guided by a small plastic shaft, the brush itself is not adherent to the wire (Boston Scientific, Boston, USA). Therefore, brush cytology could only obtained in these two patients by positioning of the brush in the tumor area, moving backward and forward in the tumor area with the tip, although a complete passage with the brush over the tumor area could not be achieved due to a rectangular path of the bile duct within the tumor. Both patients were positive for malignancy. In 4 patients material received by forceps biopsy was not suitable for diagnosis. In these patients another endoscopic procedure was performed.

RESULTS

Patient's characteristics

Patients entered into this study had the following characteristics: mean age 68 ± 10.6 years, bilirubin level 6.9 ± 7.6 mg/dL, alkaline phosphatase 559 ± 270 U/L, γ-GT 404 ± 362 U/L, and leucocytes 8.8 ± 3.8 G/L. At the time of their initial diagnosis 7/58 patients were related to bismuth stage Ⅱ, 25/58 to bismuth stage Ⅲ, and 26/58 to bismuth stage Ⅳ (Table 1). The final diagnosis was made by surgical specimens (n = 3), autopsy (n = 1), percutaneous ultrasound guided fine needle biopsy (n = 3), endoscopic ultrasound guided fine needle biopsy (n = 3), endoscopic transpapillary forceps biopsy and brush cytology (n = 35), and percutaneous transhepatic cholangioscopic guided biopsy (n = 4). In 9 patients, final diagnosis could not be confirmed by histological or cytological diagnostic methods. Therefore, diagnosis was substantiated by clinical course and survival time. Each of these 9 patients has a limited survival time ranging from 4 to 8 mo. Cholangioscopy was performed in 28 out of 58 patients. 10/29 patients had an infiltrative growth and 18/28 patients had a nodular growth.

Table 3 Sensitivity of brush cytology and forceps biopsy in patients with hilar cholangiocarcinoma n (%)

	Positive for malignancy
Brush cytology	24/73[1] (32.9)
	24/58[2] (41.4)
Forceps biopsy	31/73[1] (42.5)
	31/58[2] (53.4)
Combination of cytology and biopsy	35/73[1] (47.9)
	35/58[2] (60.3)

[1]Samples; [2]Patients.

Number of tissue sampling sessions

In case of negative results for malignancy, brush cytology and forceps biopsy was repeated. The mean number of tissue sampling sessions was 1.3 per patient. Table 2 gives an overview of the number of tissue sampling sessions. One patient of our series had 4 histological/cytological investigations. In this patient, all histological/cytological samples were negative and definite diagnosis was obtained by autopsy.

Sensitivity of endoscopic transpapillar brush cytology

Brush cytology was performed in 58 patients. Positive results for malignancy were obtained in 24/58 patients. Overall, brushing was performed 73 times in 58 patients, and malignancy was obtained in 24 of 73 cytological samples. The overall sensitivity of brush cytology for diagnosis of hilar cholangiocarcinoma was 41.4% regarding number of patients, and 32.9% regarding number of samples, respectively (Table 3).

Sensitivity of endoscopic transpapillar forceps biopsy

Forceps biopsy was performed in 58 patients. Malignancy was detected in 31 of 58 patients. If biopsy specimens were diagnosed as non-malignant, forceps biopsy was repeated up to 4 times. Overall forceps biopsy was performed 73 times in 58 patients, and was positive for malignancy in 31 of 73 histological tissue samples. Consequently, the sensitivity of forceps biopsy for the diagnosis of hilar cholangiocarcinoma was 53.4% regarding number of patients, and 42.5% regarding number of tissue samples, respectively (Table 3).

Sensitivity of brush cytology and forceps biopsy in a combined approach

The combination of the results of brush cytology and forceps biopsy resulted in a minor increase in diagnostic sensitivity to 60.3% (35/58 patients). Table 4 shows the distributions of positive and negative results for the 58 patients. 34.4% (20/58 patients) of diagnosis were obtained by both positive cytology and positive histology, 19% (11/58 patients) by positive histology (negative cytology)

Table 4 True positive and false negative results for cytological and histological investigation

	Histology positive		Histology negative		Total	
	Samples	Patients	Samples	Patients	Samples	Patients
Cytology positive	20	20	4	4	24	24
Cytology negative	11	11	38	23	49	34
Total	31	31	42	27	73	58

and only 6.9% (4/58) were confirmed by positive cytology (negative histology). Table 4 illustrates the distribution of positive and negative results of cytological and histological investigations in 73 samples.

DISCUSSION

Suspicious biliary strictures are a diagnostic challenge in endoscopic practice. Tissue collection during endoscopic retrograde cholangiography (ERC) and/or percutaneous transhepatic biliary drainage (PTBD) are the most common techniques for providing a definitive diagnosis[25]. The current study intended to determine the diagnostic value of transpapillary forceps biopsy and brush cytology in patients with hilar cholangiocarcinoma. Pugliese *et al*[25] reported sensitivity of both brush cytology and forceps biopsy in 36 patients with malignant bile duct stricture, but did not focus on hilar lesions. The patients in this study had a wide spectrum of diagnosis including pancreatic cancer (12/36), cholangiocarcinoma (10/36), intraampullary carcinoma (9/36), metastatic cancer (3/36), and malignant islet cell tumor (2/36). Cytological and histological investigation was positive in 19/36 patients. This indicates that the overall sensitivities of both techniques were almost identical (53%). In 6/10 patients (60%) with cholangiocarcinoma histology was positive, whereas cytology was positive in 7/10 patients (70%). Details regarding localization of the cholangiocarcinoma were not given in this paper. Similar to these data, Ponchon *et al*[22] also investigated a heterogeneous patient group with malignant bile duct strictures. In this series, 27/73 patients had cholangiocarcinomas, however also without specification of stricture localization. In 12/25 patients (48%) cytology, in 7/16 patients (44%) biopsy was positive for malignancy. The combination of both cytology and biopsy increased the sensitivity in 14 patients with cholangiocarcinoma to 86%.

Our study comes to the conclusion that diagnostic sensitivity is poor, since only 60.3% of diagnoses were made correctly using brush cytology and forceps biopsy in combined approaches. In our series, sensitivity of transpapillary brush cytology was 32.9% regarding number of cytological samples, and 41.4% regarding number of patients; whereas sensitivity of forceps biopsy was 42.5%, and 53.4%, respectively.

Endoscopic ultrasonography (EUS) complements the role of endoscopic and percutaneous transhepatic approaches and may provide a tissue diagnosis through fine-needle aspiration (FNA). Recently, the yield of EUS-FNA in patients with suspected cholangiocarcinoma was evaluated[26]. The authors reported a diagnostic sensitivity of 86%. However, another group reported lower rates of diagnostic sensitivity (45%) for detection of bile duct lesions by using ultrasound guided fine needle aspiration[27]. Thus, EUS-FNA may represent an alternative approach in the diagnosis of cholangiocarcinoma, especially in patients with negative brush cytology and forceps biopsy findings. One of the major limitations of endoscopic brush cytology from bile duct strictures is the poor quality of cytologic samples.

Recently, investigators have attempted to improve diagnostic assessment with an advanced cytological technique for the detection of malignant pancreaticobiliary strictures[28]. Fluorescence in situ hybridization (FISH) has shown to increase the sensitivity for the diagnosis of malignant pancreaticobiliary strictures compared to conventional cytology. Kipp *et al*[29] used a multitarget FISH probe set which has previously shown high impact in monitoring recurrent urothelial carcinoma[30]. This advanced technique identifies malignant cells by detecting aneusomy and deletion of the locus 9p21. By applying this technique for brush cytology and bile aspirate specimens in 131 patients with bile duct strictures (including 71 with primary sclerosing cholangitis, FISH analysis showed sensitivity of 35% and specificity of 91%. When patients with primary sclerosing cholangitis were excluded, sensitivity for malignancy detection by FISH was 16%[31]. This indicates that probe stets specific for biliary neoplasms will be required for higher sensitivity. However, not all malignant tumors present aneusomy or aneuploidy. In the biliary tract, the percentage of cancers displaying aneuploidy has been estimated to be approximately 80%[32]. Since our current data clearly demonstrate that no improved sensitivity can be obtained by using forceps biopsy and/or brush cytology during endoscopic procedures, we therefore suggest that additional diagnostic techniques such as FISH techniques should be routinely evaluated in patients with negative cytological and histological findings.

COMMENTS

Background

Surgery is the only curative treatment in patients with hilar cholangiocarcinoma. The results are more favourable for patients with early-stage disease. Therefore, a reliable diagnostic procedure is of great importance in these patients. Cholangiocarcinomas often grow longitudinally along the bile duct rather than in radial direction away from the bile duct. Consequently, imaging techniques including ultrasound, computed tomigraphy (CT) and magnetic resonance imaging (MRI) are of limited sensitivity for the detection of cholangiocarcinoma. In patients with suspected malignant biliary stricture brush cytology and/or forceps biopsy were routinely used for distinction between benign and malignant strictures. Biliary tissue collection provides the only definitive diagnosis that can be used for establishing therapeutic strategies.

Research frontiers

To our knowledge, no study exists investigating the sensitivity of brush cytology and forceps biopsy in a homogeneous group of patients with hilar cholangiocarcinoma. The current study was designed to address the question of diagnostic sensitivity for endoscopic transpapillary brush cytology and forceps biopsy in 58 patients with hilar cholangiocarcinoma.

Innovations and breakthroughs

Positive results for malignancy were obtained in 41.4% of patients using brush cytology and in 53.4% of patients using forceps biopsy. The combination of both techniques brush cytology and forceps biopsy resulted only in a minor increase in diagnostic sensitivity to 60.3%. Consequently, in 23 out of 58 patients definitive diagnosis could not achieved using the combination of brush cytology and forceps biopsy.

Applications

Brush cytology and forceps biopsy have only limited sensitivity for the definite diagnosis of hilar cholangiocarcinoma. In our eyes, additional diagnostic techniques such as FISH techniques should be further evaluated and should become routine in patients with negative cytological and histological findings.

Terminology

Cholangiocarcinomas are categorized as intrahepatic and extrahepatic carcinomas. Extrahepatic cholangiocarcinomas are further subdivided into hilar, medial and distal cholangiocarcinomas. The most common type is hilar cholangiocarcinoma which is also called Klatskin tumor. Klatskin tumors are classified into 4 stages according to the bismuth classification: stage Ⅰ or Ⅱ for tumors expanding up the hilus, type Ⅲ infiltrating right (ⅢA) or left (ⅢB) hepatic duct, and stage Ⅳ with infiltration of both hepatic ducts and sub segments.

Peer review

The authors retrospectively analyze the effectiveness of various diagnostic tools to diagnose hilar cholangiocarcinoma. The authors come to the conclusions that brush cytology and forceps biopsy have only limited sensitivity for the definite diagnosis of hilar cholangiocarcinoma. The study suggest that in patients with suspected biliary stricture and negative cytological / histological findings further diagnostic approaches such as cholangioscopy should performed.

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RAPID COMMUNICATION

Effect of preoperative biliary drainage on surgical results after pancreaticoduodenectomy in patients with distal common bile duct cancer: Focused on the rate of decrease in serum bilirubin

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Abstract

AIM: To examine if the rate of decrease in serum bilirubin after preoperative biliary drainagecan be used as a predicting factor for surgical complications and postoperative recovery after pancreaticoduodenectomy in patients with distal common bile duct cancer.

METHODS: A retrospective study was performed in 49 consecutive patients who underwent pancreaticoduodenectomy for distal common bile duct cancer. Potential risk factors were compared between the complicated and uncomplicated groups. Also, the rates of decrease in serum bilirubin were compared pre- and postoperatively.

RESULTS: Preoperative biliary drainage (PBD) was performed in 40 patients (81.6%). Postoperative morbidity and mortality rates were 46.9% (23/49) and 6.1% (3/49), respectively. The presence or absence of PBD was not different between the complicated and uncomplicated groups. In patients with PBD, neither the absolute level nor the rate of decrease in serum bilirubin was significantly different. Patients with rapid decrease preoperatively showed faster decrease during the first postoperative week (5.5 ± 4.4 μmol/L *vs* -1.7 ± 9.9 μmol/L, $P = 0.004$).

CONCLUSION: PBD does not affect the surgical outcome of pancreaticoduodenectomy in patients with distal common bile duct cancer. There is a certain group of patients with a compromised hepatic excretory function, which is represented by the slow rate of decrease in serum bilirubin after PBD.

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Key words: Distal bile duct cancer; Drainage; Bilirubin; Rate

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INTRODUCTION

In spite of recent advances in surgical techniques and perioperative management, pancreaticoduodenectomy still carries a significant morbidity and mortality[1,2]. Many studies have been performed to identify the high risk patients for the operation with inconsistent results. The diversity of those study results may be due in part to the heterogeneity of the disease entities included, and also to the patient's conditions which in turn affect the outcome of the complex operative procedures. One of the prime interests has been the effectiveness of preoperative biliary drainage. Many studies favored the usefulness of biliary drainage in improving the surgical outcome[3-7], while others questioned its effectiveness[8-10], and even concluded it provokes more complications[11-15]. The underlying rationale for the preoperative decompression of the bile duct is that obstructive jaundice may interfere with the hepatic function, and relieving the obstruction could improve the recovery of the patients and hence the surgical results[16,17]. It was our impression that there are a certain group of patients whose concentration of total serum bilirubin drops faster after biliary drainage than others. Presumably, the more slowly the concentration of

serum bilirubin drops, the higher the risk of developing complications following pancreaticoduodenectomy might be. If the surgical outcome is complicated by obstructive jaundice, this should be measured by not only the absolute concentration of serum bilirubin but also its rate of decrease after biliary drainage, which is the indicator for hepatic excretory dysfunction and further the presence of liver damage[18-21].

We conducted this study to examine whether the rate of decrease in the concentration of serum bilirubin, as well as its absolute preoperative level per se, are different between the patients with and without major complications after pancreaticoduodenectomy. To avoid skewed results by confounding variables, we included patients with distal common bile duct cancer only.

MATERIALS AND METHODS

Study patients and data collection

A retrospective chart review was done in 49 consecutive patients who received pancreaticoduodenectomy for distal common bile duct cancer from July 1996 to June 2005. Patients were divided into complicated and uncomplicated groups according to whether the complications occurred. We compared the potential risk factors including demographic data, laboratory values, operative data and the history of preoperative biliary drainage between the two groups. Then, the patients with biliary drainage were divided further into two arbitrary subgroups with same sample size according to the rate of decrease in the concentration of serum bilirubin after preoperative biliary drainage, and the incidence of postoperative complications and the postoperative decrease in serum bilirubin were compared between the two subgroups.

Regarding the total serum bilirubin level, we used six parameters defined as follows: (1) the serum bilirubin at presentation as the initial bilirubin (Bi), (2) the preoperative concentration of serum bilirubin (Bo), (3) the ratio of decrease in serum bilirubin [(Bi-Bo)/Bi], (4) the rate of decrease in serum bilirubin per day [(Bi-Bo)/(days of biliary drainage)], (5) the rate of decrease in serum bilirubin during the first week following surgery (Bpod1), (6) the rate of decrease in serum bilirubin during the second week following surgery (Bpod2).

Preoperative biliary drainage

No absolute criterion existed for performing the preoperative biliary drainage. In general, when there were suspicions as to the nature of obstructive jaundice such as benign stricture and choledocholithiasis or malignant obstruction, endoscopic retrograde cholangiography (ERC) was performed for both diagnostic and therapeutic purposes. After the ERC, the patients were usually followed by endoscopic nasobiliary drainage (ENBD) to avoid secondary cholangitis. When malignant jaundice was strongly suspected, magnetic resonance cholangiography (MRC) was performed rather than ERC, in that MRC is a less invasive procedure than ERC. After the presence of malignant biliary obstruction was confirmed, the patients were either followed by percutaneous transhepatic biliary drainage (PTBD) or surgery at surgeon's discretion. The absolute level of serum bilirubin was not regarded as a criterion for the timing of operation. In cases in which the preoperative biliary drainage was considered to be inadequate, in terms of the small daily drainage amount or suspicions for displacement, either the drainage was replaced or the operation schedule was advanced.

Operative procedures

The resection phase of pancreaticoduodenectomy consisted of the standard operative procedures including the regional lymphnode dissection of the porta hepatis, retropancreatic and celiac artery area. When the tumor was suspected to be advanced locally, the distal stomach was resected and the procedure was directed to the standard Whipple's operation. Otherwise, the pylorus was preserved at the pylorus-preserving pancreaticoduodenectomy. The pancreatico-enteric anastomosis was done by either end-to-side pancreaticojejunostomy or duct to mucosa technique. Two active suction drains were applied at the pancreatico-enteric anastomosis site.

Definition of complications

Surgical mortality was defined as the death noted within 30 d postoperatively. Postoperative complications included delayed gastric emptying, pancreatic fistula, intraabdominal abscess and postoperative bleeding. Systemic complications such as pulmonary and cardiac complications were also noted. Delayed gastric emptying was defined as the inability to resume oral intake of food for more than 10 d postoperatively without the evidence of mechanical bowel obstruction. Pancreatic fistula was defined as a drain output more than 10 mL per day of amylase-rich fluid after postoperative day 8 or for more than 8 days postoperatively[22,23]. An intraabdominal abscess was present when there was pus collected intraperitoneally causing symptoms that warranted either surgical or interventional drainage. Postoperative bleeding was defined as a significant drop in hemoglobin concentration with the evidence of intraluminal and/or extraluminal bleeding that required re-exploration or interventional bleeding control. Minor complications such as wound infection that did not affect the overall recovery were not included in the complication.

Statistical analysis

For nominal variables, either χ^2-test or Fisher's exact test was applied where appropriate, and Mann-Whitney *U* test was used for interval variables. Because of the limited number of cases, multivariate analysis was considered inappropriate and not performed. Statistical analysis was done using SPSS for Windows Ver. 12.0.1 (SPSS Inc., Chicago, Illinois). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Demographics of the 49 patients are summarized in Table 1. A total of 26 patients (53.1%) with no major complications were assigned to the uncomplicated group. The remaining 23 patients (46.9%) with major complications were classified as complicated group. In the complicated group, 3 patients

Table 1 Patient characteristics *n* (%)

Demographics	Total (*n* = 49)	Uncomplicated (*n* = 26)	Complicated (*n* = 23)	*P*
Age (yr)	64.0 ± 8.9	62.0 ± 9.9	65.5 ± 7.3	0.228
Sex				0.475
Male	36 (73.5)	18 (69.2)	18 (78.3)	
Female	13 (26.5)	8 (30.8)	5 (21.7)	
Diabetes				0.483
Yes	10 (20.4)	4 (15.4)	6 (26.1)	
No	39 (79.6)	22 (84.6)	17 (73.9)	
Operation				0.879
Whipple procedure	24 (49.0)	13 (50.0)	11 (47.8)	
PPPD	25 (51.0)	13 (50.0)	12 (52.2)	
Biliary drainage				0.472
Yes	40 (81.6)	20 (76.9)	20 (87.0)	
No	9 (18.4)	6 (23.1)	3 (13.0)	
[1]Leukocytosis				1.000
Yes	4 (8.2)	2 (7.7)	2 (8.7)	
No	45 (91.8)	24 (92.3)	21 (91.3)	
Bilirubin (μmol/L)	116.3 ± 92.3	107.7 ± 92.0	124.8 ± 97.5	0.652
Albumin (g/L)	34.2 ± 4.3	34.0 ± 4.4	34.4 ± 4.4	0.912
Creatinine (μmol/L)	70.7 ± 17.7	70.7 ± 26.5	70.7 ± 17.7	0.481
Alkaline phosphatase (U/L)	601 ± 450	632 ± 544	565 ± 322	0.764
Hemoglobin (g/L)	121.0 ± 16.5	117.8 ± 14.6	124.7 ± 18.0	0.193
Hematocrit (%)	35.6 ± 4.8	34.8 ± 4.3	36.4 ± 5.3	0.307
Operation data				
Red cell transfusion (mL)	522 ± 401	442 ± 358	613 ± 435	0.116
Operation time (min)	515 ± 107	510 ± 104	520 ± 111	0.825

[1]Defined as leukocyte count more than $10.8 \times 10^3/mm^3$. All laboratory values are preoperative serum levels expressed as mean ± SD, unless specified otherwise. PPPD: Pylorus preserving pancreaticoduodenectomy.

Table 2 Surgical morbidity and mortality *n* (%)

Morbidity and mortality	Number of patients
[1]Morbidity	23 (46.9)
Bleeding	4 (8.2)
Intraabdominal abscess	6 (12.2)
Pancreatic fistula	7 (14.3)
Delayed gastric emptying	7 (14.3)
Pulmonary complication	2 (4.1)
Mortality	3 (6.1)
Bleeding, DIC	2 (4.1)
Intraabdominal abscess, sepsis	1 (2.0)

[1]A patient may have more than one complication. DIC: Disseminated intravascular coagulation.

(6.1%) died of complications. A detailed description of the morbidity and mortality is presented in Table 2. On univariate analysis, none of the age, sex, preoperative laboratory values and operative data was statistically different between the two groups.

In our series, 40 patients (81.6%) underwent preoperative biliary drainage. There was no difference in the preoperative bilirubin level whether or not the biliary drainage procedure was performed between the uncomplicated and complicated groups (76.9% *vs* 87.0%, respectively, $P = 0.472$), although the preoperative bilirubin level was significantly lower in those patients without biliary drainage than in those with biliary drainage (41.0 ± 45.8 *vs* 132.2 ± 93.0 μmol/L, respectively, $P = 0.001$). The parameters related to the biliary drainage are shown in Table 3. ENBD was performed in 29 patients (72.5%) and PTBD in 11 patients (27.5%). The method of drainage was not different between the two groups. The initial, preoperative total serum bilirubin level or the duration of drainage was not different between the two groups. Also, neither the ratio nor the rate of decrease in serum bilirubin showed a statistically significant difference ($P = 0.892$ and 0.425, respectively), although there was a tendency for the rate of decrease in serum bilirubin to be higher in the uncomplicated group (Figure 1). The bilirubin drop during the first and second postoperative weeks was not different between the two groups. When the patients were regrouped according to the rate of decrease in serum

Table 3 Analysis of preoperative biliary drainage-related factors *n* (%)

Factors	Total (*n* = 40)	Uncomplicated (*n* = 20)	Complicated (*n* = 20)	*P*
Drainage methods				0.723
ENBD	29 (72.5)	15 (75.0)	14 (70.0)	
PTBD	11 (27.5)	5 (25.0)	6 (30.0)	
Initial bilirubin (Bi) (μmol/L)	229.1 ± 135.1	210.3 ± 157.3	246.2 ± 111.2	0.223
Preop. bilirubin (Bo) (μmol/L)	131.7 ± 92.3	123.1 ± 94.1	140.2 ± 94.1	0.552
Duration of drainage (d)	10 ± 9	8 ± 5	12 ± 12	0.392
Decrease in bilirubin (Bi–Bo, dB, μmol/L)	95.8 ± 104.3	87.2 ± 94.1	104.3 ± 114.6	0.715
Ratio of decrease in bilirubin (dB/Bi)	0.37 ± 0.35	0.37 ± 0.31	0.38 ± 0.40	0.892
Rate of decrease in bilirubin (dB/d, μmol/L)	10.1 ± 16.6	13.2 ± 14.5	7.0 ± 18.1	0.425
Bpod1 (μmol/L)	1.9 ± 8.6	4.4 ± 5.4	-0.6 ± 10.3	0.180
Bpod2 (μmol/L)	2.2 ± 9.1	4.4 ± 5.8	-0.1 ± 11.2	0.228

Variables are presented as mean ± SD, unless specified otherwise. ENBD: Endoscopic nasobiliary drainage; PTBD: Percutaneous transhepatic biliary drainage; Bpod1: Decrease in serum bilirubin during the first postoperative week; Bpod2: Decrease in serum bilirubin during the second postoperative week.

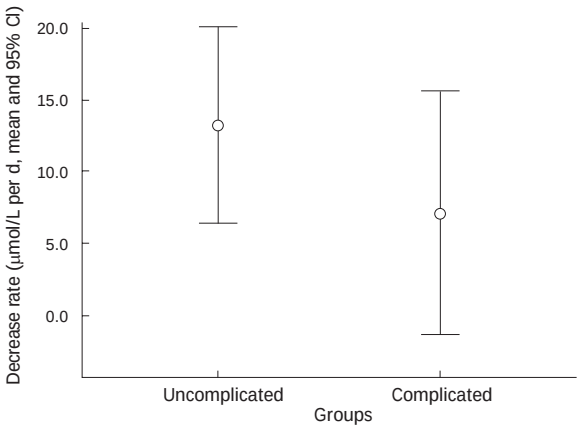


Figure 1 Rate of decrease in serum bilirubin comparing the uncomplicated and complicated groups (CI: Confidence interval).

Table 4 Rate of decrease in serum bilirubin after preoperative biliary drainage *n* (%)

Factors	Total (*n* = 40)	Fast decrease group (*n* = 20)	Slow decrease group (*n* = 20)	*P*
Drainage methods				0.723
ENBD	29 (72.5)	14 (70)	15 (75)	
PTBD	11 (27.5)	6 (30)	5 (25)	
Complication				0.527
Uncomplicated	20 (50)	11 (55)	9 (45)	
Complicated	20 (50)	9 (45)	11 (50)	
Bpod1 (μmol/L)	1.9 ± 8.6	5.5 ± 4.4	-1.7 ± 9.9	0.004
Bpod2 (μmol/L)	2.2 ± 9.1	1.9 ± 4.3	2.4 ± 12.3	0.818

Variables are presented as mean ± SD, unless specified otherwise. ENBD: Endoscopic nasobiliary drainage; PTBD: percutaneous transhepatic biliary drainage; Bpod1: decrease in serum bilirubin during the first postoperative week; Bpod2: decrease in serum bilirubin during the second postoperative week.

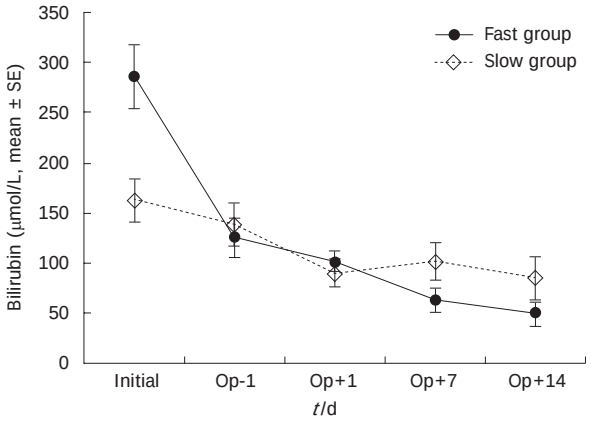


Figure 2 Changes in serum bilirubin comparing the fast and slow decrease groups (Op: Operation).

bilirubin after biliary drainage, those with a rapid decrease preoperatively showed a significantly faster decrease during the first postoperative week as well, than those with a slower decrease preoperatively ($P = 0.004$). This difference was not seen during the second postoperative week (Table 4, Figure 2).

DISCUSSION

The effect of preoperative biliary drainage in preventing the operative complications after pancreaticoduodenectomy has been addressed in patients with obstructive jaundice due to periampullary tumors. To date, many studies have examined the issue with inconsistent results[10-12]. Periampullary tumors include various benign tumorous conditions such as chronic pancreatitis occurring at the periampullary region, in addition to the malignant lesions developed in the pancreas head, distal common bile duct, ampulla of Vater and duodenum. While the malignant diseases developing at the periampullary region have many characteristics in common, the biologic behavior of each is believed to be different from one another[24], which may in turn affect the surgical results. Also, the standard surgical procedure of pancreaticoduodenectomy is a complicated one with diverse technical variations, and the choice of reconstruction method and the extent of dissection may be dependent on both the operator and the disease entity. In analyzing the effect of preoperative biliary drainage, these factors are intermingled with each other, making the analysis difficult. To minimize the confounding variables, the present study limited the disease entity to the distal common bile duct cancers only. The rationale of selecting distal common bile duct cancers among the other periampullary malignancies is that these cancers are usually fairly confined within the periampullary region compared to the more aggressive diseases such as the pancreas head cancers, while the volume of cases in our database is large enough to make the statistical analysis valid. Nevertheless, it is not so large as to satisfy the requirements for the multivariate analysis, and we performed the univariate analysis only.

Many reports showed that the effectiveness of preoperative biliary drainage is measured by the surgical outcome following pancreaticoduodenectomy according to the specific complication items[8,9,14,15]. The surgical morbidities are commonly classified as bleeding, pancreatic leakage, intraabdominal abscess, delayed gastric emptying and others which include wound infection and systemic complications such as pneumonia or cardiac problems. While the complications are distinct entities clinically, the causes for each complication are either interrelated or obscure, and may be related to the basic fundamental defect, i.e. pancreaticojejunostomy failure[25-27]. Postoperative hemorrhage, when clinically significant, usually originates from the rupture of pseudoaneurysm, which in turn develops most frequently from the inflammatory erosion of arterial wall due to the leaked pancreatic juice[28]. Intraabdominal abscess can be caused by any leakage from the anastomoses, and the pancreaticoenteric anastomosis is the most frequent one to leak[27]. Also, the underlying mechanism of delayed gastric emptying is still unclear, but many authors suggest that the local inflammation induced by the leaked pancreatic enzymes may play an important role[27,29]. Some of the studies performed to date have classified the surgical results as complicated and uncomplicated[7,10-12]. This simple classification of surgical outcome measurement is advantageous in controlling as many confounding variables as possible. Surely, this simplified approach may not identify the risk factors predisposing the specific complications. However, based on the limited number of cases in the current study, it is more plausible to classify the surgical results as complicated and uncomplicated. In essence, this study was designed to investigate if the relief of obstructive jaundice preoperatively can reduce the incidence of the pancreatic anastomosis failure after pancreaticoduodenectomy.

Obstructive jaundice can cause tissue damage by various inflammatory mediators including oxygen free radicals[30]. Animal experiments suggested that the inflammatory mediators have a deleterious effect on not only the hepatic sinusoidal endothelium and then the hepatic function, but also the functions of other remote organs[31,32]. Both clinical and experimental studies have also shown that obstructive jaundice impairs the healing of

abdominal wounds[33], although there is still a controversy in this series[17]. Arnaud *et al*[34] observed that the healing of gastric wounds and abdominal wounds, is delayed in experimentally jaundiced animals. Also, Takahashi[35] showed that the bursting strength of pancreaticojejunostomy is higher and the incidence of anastomotic leakage is lower in dogs with their concentration of serum bilirubin < 5 mg/dL. Considering such evidence, it seems prudent to assume that the high concentration of serum bilirubin is closely associated with the impaired healing of intestinal anastomoses, although the relevant mechanism and the magnitude of such association are still unclear.

Some studies have examined whether the relief of obstructive jaundice could improve the healing of anastomotic wound[36]. Vendemiale *et al*[16] showed that the impairment of hepatic redox status caused by cholestasis in patients with extra-hepatic biliary obstruction could be almost completely resolved by surgical biliary drainage. In addition, Koivukangas *et al*[17] observed that the decreased baseline collagen synthesis in jaundiced patients is partly restored by the resolution of jaundice, although the healing of the blister wound is not affected.

In spite of the scattered evidence that favors the possible positive effect of preoperative biliary drainage on the operative results after pancreaticoduodenectomy, many studies performed so far have failed to show any preferable impact. Povoski *et al*[11] have extensively reviewed the effectiveness of biliary drainage reported in the literature with their own experiences and concluded that the preoperative biliary drainage has no beneficial effect on the surgical results, and rather increases the infectious complications following pancreaticoduodenectomy. In another meta-analysis, Sewnath *et al*[10] also concluded that the preoperative biliary drainage prolongs hospital stay and has its own procedure-related complications without any proven positive effect on immediate postoperative results.

It is our experience that there is a certain group of patients whose serum bilirubin level decrease faster after biliary drainage procedures while there are others in whom the serum bilirubin fails to decrease effectively or even increases despite of good-functioning drainage route. We assume that if the surgical outcome is complicated by obstructive jaundice, this can be measured by not only the absolute serum bilirubin level[18,19], but also its rate of decrease after biliary drainage which is the indicator for hepatic excretory dysfunction and further the presence of liver damage. To date, a few studies have focused on the rate of decrease in the concentration of serum bilirubin[9,20,21]. Even though these studies may have their own limitations, such as the limited number of cases or the inclusion of heterogeneous disease entities, they have shown that the postoperative complications are more prone to develop in patients whose rate of decrease in the level of serum bilirubin is slower after drainage[20,21].

We used two indicators for the changes in the concentration of serum bilirubin: the ratio and rate. None of these indicators showed any statistically significant difference between the complicated and uncomplicated groups, although the serum bilirubin in the uncomplicated group tended to decrease faster than that in the complicated group after biliary drainage. To further investigate the postoperative recovery of hepatic excretory function, we divided the patients into two subgroups according to the rate of decrease in serum bilirubin after drainage. Patients with a higher rate of decrease in the concentration of serum bilirubin did not show a significantly lower rate of postoperative complications. In these patients, however, the rate of decrease in serum bilirubin was also higher during the first postoperative week. These results indicate that the rate of decrease in serum bilirubin after drainage can predict the immediate postoperative hepatic excretory functional recovery, although it cannot select high risk patients. Our results also show that there was a certain group of patients with their hepatic excretory function already compromised by obstructive jaundice before it was relieved by surgical resection of the causative lesion. These findings are in agreement with those presented by Sano *et al*[21], who showed that biliary obstruction causes liver damage represented by the slower rate of serum bilirubin decrease in patients who underwent biliary drainage.

Our study could not disclose the relationship between the compromised hepatic excretory function and surgical morbidity of pancreaticoduodenectomy. We think that the negative results may be due to the limited number of cases. Well-designed, prospective randomized studies in a larger number of cases are warranted to assess the clinical usefulness of the serum bilirubin decrease rate in selecting high risk patients to whom greater attention must be paid during surgery.

COMMENTS

Background

In spite of recent advances in surgical techniques and perioperative management, pancreaticoduodenectomy still carries a significant morbidity and mortality. Many studies have been performed to identify the high risk patients for the operation with inconsistent results. One of the prime interests has been the effectiveness of preoperative biliary drainage, but the issue is under debate.

Research frontiers

Many studies favored the usefulness of preoperative biliary drainage in improving the surgical outcome, while others questioned its effectiveness, and even concluded it provokes more complications. Further studies are warranted on the subject.

Innovations and breakthroughs

If the surgical outcome is complicated by obstructive jaundice, this should be measured by not only the absolute concentration of serum bilirubin but also its rate of decrease after biliary drainage, which is the indicator for hepatic excretory dysfunction and further the presence of liver damage. So far, few studies have focused on the rate of decrease in serum bilirubin.

Applications

We conducted this study to examine whether the rate of decrease in the concentration of serum bilirubin, as well as its absolute preoperative level per se, is different between the patients with and without major complications after pancreaticoduodenectomy. To avoid skewed results by confounding variables, we included patients with distal common bile duct cancer only.

Peer review

The study has certain merits since the authors consider several different variables for each patient. Also, the manuscript is easy to follow and its content is rich.

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RAPID COMMUNICATION

Ursodeoxycholic acid treatment improves hepatocyte ultrastructure in rat liver fibrosis

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Abstract

AIM: To examine the ultrastructural changes after ursodeoxycholic acid (UDCA) treatment in hepatocytes from experimentally induced fibrotic livers.

METHODS: Liver fibrosis was induced in male Sprague-Dawley rats with CCl_4 for 12 wk, and the rats were divided into two groups. Group Ⅰ was treated with saline and group Ⅱ with UDCA (25 mg/kg per day) for 4 wk. All the rats were killed at wk 16. Mitochondria, nuclei, rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) of hepatocytes were evaluated according to a scoring system.

RESULTS: Mitochondria, nuclei, RER and SER injury scores in group Ⅱ were significantly lower than those in group Ⅰ ($P < 0.001$).

CONCLUSION: UDCA alleviates hepatocyte organelle injury in CCl_4-induced liver fibrosis.

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Key words: Rat liver fibrosis; Rat liver cirrhosis; Ursodeoxycholic acid; Electron microscopy; Hepatocyte ultrastructure

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INTRODUCTION

Liver fibrosis is characterized by an increase in extracellular matrix (ECM) components and occurs as a common sequel of various chronic inflammatory diseases. Certain collagen types deposit around the sinusoidal cell layer in the space of Disse and a molecular re-organization of various ECM molecules results in alterations in the composition of the fibrotic matrix, with deterioration of hepatic functions[1]. Parenchymal cell damage of varying etiology is central to the initiation of fibrogenesis in the liver. Although experimental and clinical trials to establish effective options for prevention or regression of fibrogenesis in the liver are being conducted, it is still an untreatable condition. Some agents that are currently used for other diseases, and several herbal drugs, have recently been reported to have antifibrotic efficacy with different potency in the liver[2].

Ursodeoxycholic acid (UDCA) is the mainstay of therapy of primary biliary cirrhosis (PBC) in humans. UDCA has been reported to have antifibrotic activity in bile duct ligation and CCl_4-induced experimental liver fibrosis[3,4]. Clinically, long-term UDCA therapy improves liver function tests[5] and delays the development and progression of cirrhosis in PBC[6,7], which leads to prolongation of the time needed for liver transplantation. It has been reported to have antiapoptotic activity on cholangiocytes and hepatocytes[8,9] and an inhibitory effect on generation of reactive oxygen species[10]. In the present study, we examined the ultrastructural changes after UDCA treatment in hepatocytes from experimentally induced liver fibrosis in the rat. This should show us alterations in hepatocyte structure after treatment of established fibrosis and the possibility of regression of organelle injury.

MATERIALS AND METHODS

The Institutional Animal Use and Care Committee of the Gülhane Medical Academy, Ankara, Turkey approved the study, which was also performed in accordance with the National Institute of Health's guidelines for the care and handling of animals. The animals were fed standard rat chow and kept in metabolic cages with controlled temperature and a 12-h light/dark cycle before and during the experiment.

Twenty male Sprague-Dawley rats weighing 250-350 g were injected subcutaneously with 0.2 mL/100 g CCl_4 twice weekly for 12 wk to induce liver fibrosis. Then, the

Table 1 Ultrastructural scoring system

Nucleus	
0:	Normal
1:	Irregular chromatin distribution
	-Margination
	-Clumping
2:	Increased heterochromatine
3:	Degenerated nucleus
Mitochondria	
0:	Normal
1:	Prominent cristae
2:	Swelling
3:	Collection of amorphous material
RER	
0:	Normal
1:	Dilatation
2:	Irregular lamellar organization
3:	Presence of focal breaks
SER	
0:	Normal
1:	Dilatation
2:	Presence of vacuole formation (vacuolization)
3:	Presence of focal breaks

rats were randomly divided into two groups. Group I ($n = 10$) was treated with saline and group Ⅱ ($n = 10$) with UDCA (25 mg/kg per day) for 4 wk. At the end of wk 16, all the rats were killed under anesthesia and the livers were excised under sterile conditions. The specimens were fixed in 2.5% glutaraldehyde for 24 h and consequently washed in phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7.4), and dehydrated in increasing concentrations of alcohol. The tissues were washed with propylene oxide and embedded in epoxy-resin embedding medium. Semi-thin sections approximately 60-90 nm in thickness were cut with a glass knife on an LKB Nova (Sweden) ultramicrotome. Sections were stained with methylene blue and examined by a Nikon Optiphot (Japan) light microscope. Ultrathin 60-90 nm sections were collected on copper grids, stained with uranyl acetate and lead citrate, and examined with a Jeol JEM 1200 Ex (Japan) transmission electron microscope. Twenty cells from each specimen were examined. Mitochondria, nuclei, rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) of hepatocytes were examined according to a previously described scoring system[11,12] (Table 1).

Results were expressed as means ± SEM. The significance of differences in histopathological and ultrastructural fibrosis scores and serum amylase activity were assessed by the Kruskal-Wallis test. $P < 0.05$ was considered significant.

RESULTS

After 4 wk UDCA treatment (by the end of wk 16), the most remarkable finding under electron microscopic examination of tissues from saline-treated animals (group Ⅰ) was irregular lamellar organizations of the RER. Large dilatations and focal breaks in the RER were also found in many areas (Figure 1A). Vacuolization in the SER and presence of myelin figures were the other prominent findings in saline-treated animals (Figure 1B). Many of the nuclei had irregular chromatin distribution and margination with clumping. An increase in heterochromatin was seen in some of the nuclei. Almost all of the mitochondria had prominent swelling that was much more severe than that in the UDCA-treated group. Another important finding was the collection of active fibroblasts in some focal areas of the liver.

Ultrastructural findings in the UDCA-treated group (group Ⅱ) were margination and clumping of the chromatin and a marked increase in the amount of heterochromatin (Figure 1C). Mild swelling of mitochondria was observed (Figure 1D). There were mild dilations in the SER and Golgi apparatus. However, vacuole formation in the SER was seen in only a few areas. There were also some breaks in the RER (Figure 1D and E).

When these findings were scored and evaluated according to the system shown in (Table 1), we found that mitochondria (44 ± 0.5 *vs* 61 ± 2, $t = 7.38$, $P < 0.001$), nuclei (29 ± 0.8 *vs* 46 ± 1.5, $t = 9.3$, $P < 0.001$), RER (30 ± 0.7 *vs* 56 ± 1, $t = 21.7$, $P < 0.001$), and SER (22 ± 0.5 *vs* 42 ± 1.2, $t = 15.3$, $P < 0.001$), and injury scores in group Ⅱ were significantly improved. Total electron microscopy score in group Ⅱ was also significantly lower when compared to that in group I (125 ± 2.3 *vs* 205 ± 3.7, $t = 18.2$, $P < 0.001$) (Figure 2).

DISCUSSION

The hepatocytes are the parenchyma cells of the liver, whose main function is synthesis of vital substances and removal of various kinds of molecules from the blood and their excretion into bile. Liver fibrosis is the result of deterioration of function of these cells, which results in initiation of many pathogenic events in the liver. As a result of parenchymal cell damage, activation of hepatic stellate cells occurs, which results in excessive production of collagen, and ultimately pathological and clinical features of cirrhosis develop[13].

UDCA has been shown to reduce hepatocellular damage in PBC. Treatment of patients with PBC with UDCA prevents deposition of perisinusoidal collagen[14]. It prevents hepatocyte apoptosis *in vitro* by inhibiting mitochondrial membrane permeability transition[15]. We performed this work to identify the ultrastructural changes in individual organelles of hepatocytes from UDCA-treated fibrotic livers. Histological changes in hepatic structure after CCl_4 treatment have been well documented. Indeed, most studies addressing antifibrotic efficacy of newly tested agents have been supported by direct evidence of histological changes, despite clear biochemical findings. However, electron microscopically supported morphological results are not frequent. Although there have been some ultrastructural studies of the effects of UDCA on the canalicular system, organelle-based activity of this antifibrotic agent may be helpful.

Although the liver has a high regeneration capacity, which can be linked to the phenomenon of spontaneous recovery, we still found severe hepatocyte organelle injury

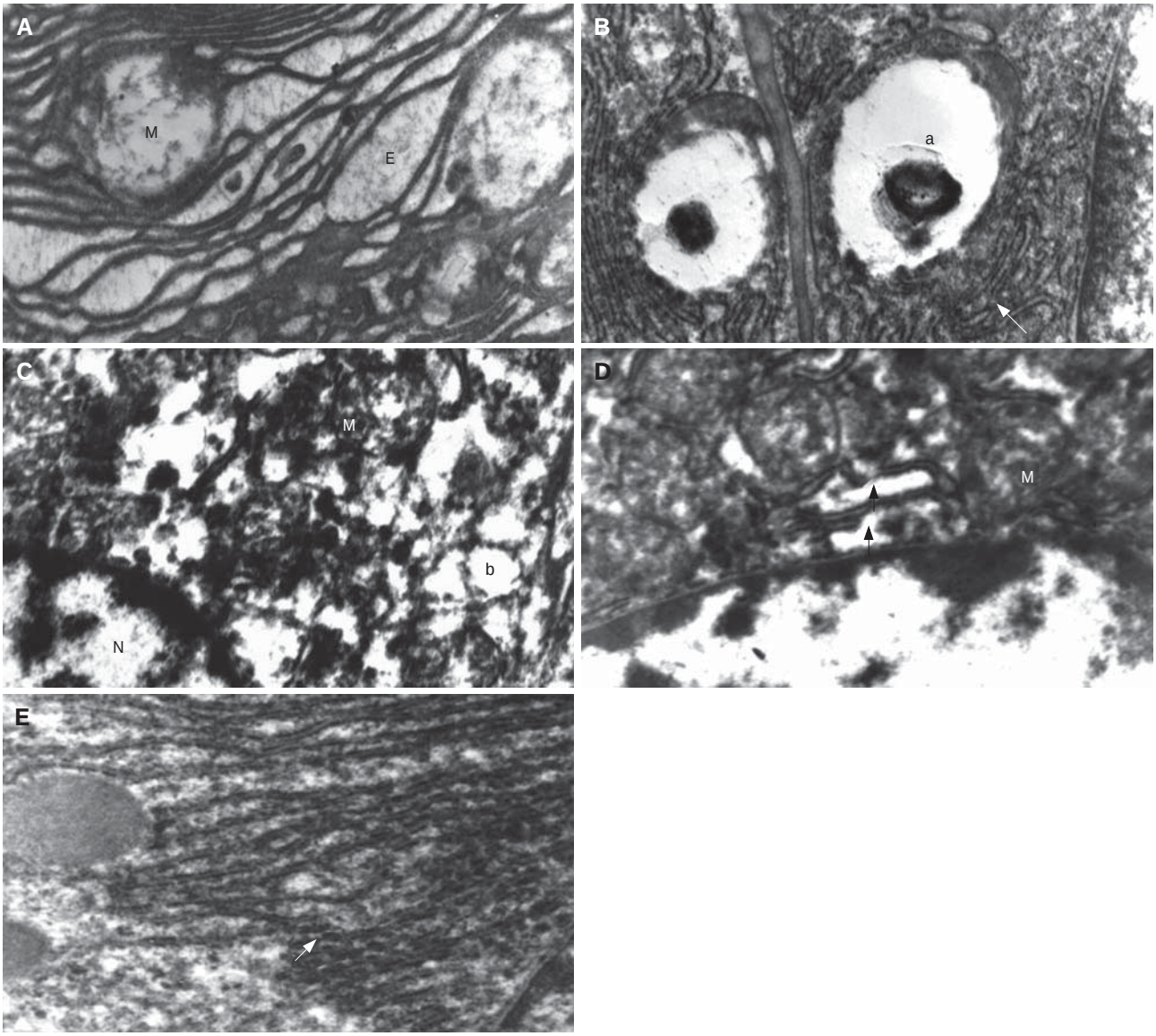


Figure 1 **A**: Electron micrograph showing irregular lamelar organizations and dilation of the RER (E) and swollen mitochondria (M) in the CCl_4 group (× 15 000); **B**: Electron micrograph showing myelin figures in the SER (a) and breaks in the RER (arrow) in group Ⅰ (× 20 000); **C**: Electron micrograph showing the margination and clumping of the chromatin in the nucleus (N), mild swelling in a mitochondrion (M), mild dilation in the SER (b) and breaks in RER in group Ⅱ (× 15 000); **D**: Electron micrograph showing mild swelling in the mitochondria (M) and breaks in the RER (arrow) in group Ⅱ (× 20 000); **E**: Electron micrograph showing normal RER (arrow) in group Ⅱ (× 20 000).

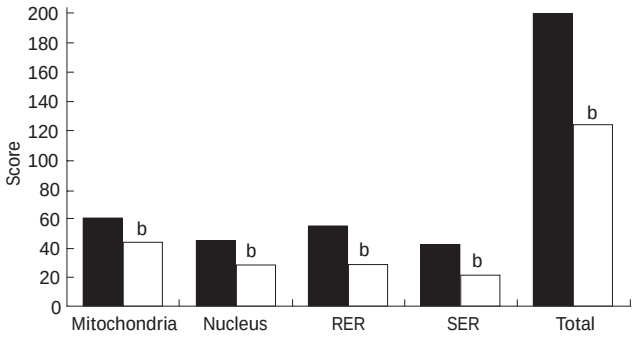


Figure 2 Ultrastructural injury scores in hepatocytes. Individual and total organelle injury scores decreased significantly in UDCA-treated compared with saline-treated animals. $^bP < 0.001$.

after 4 wk of saline administration in CCl_4-treated animals. Disrupted organization of the RER, vacuolization of the SER, and disorganization of nuclear content were indicative of severe organelle damage and established fibrosis. Munoz *et al* have reported a similar organization of ER with CCl_4 treatment in their study which examined the protective effect of adenosine on hepatotoxin-induced liver fibrosis[16]. They have also reported mitochondrial swelling and breaks in the ER after CCl_4 treatment.

Altered chromatin distribution in the nuclei was another noticeable finding in the hepatocytes following hepatotoxin exposure, which is suggestive of nuclear events taking place during necrosis and/or apoptosis. Heterochromatin is the condensed form of chromatin, with specific molecular characteristics and nuclear functions. It occurs around the centromeres and is involved in controlling the transcriptional state of the genome by gene silencing and nuclear organization. It lies next to the inner side of the

nuclear envelope and is broken up at the sites of nuclear pores. Abundant heterochromatin is seen in resting cells, whereas euchromatin is mostly found in transcriptionally active cells. We found a significant decrease in heterochromatin after 4 wk of UDCA treatment. This may be intranuclear morphological evidence of improving regular cell functions. Mitochondrial swelling, which is characteristic of deteriorated function of this organelle, decreased significantly with UDCA. Taken together with parallel changes seen in the nucleus, we could not conclude any association of this finding with apoptosis, although, antiapoptotic activity of UDCA on hepatocytes has previously been demonstrated[9]. Structural reversal of disrupted ER may also result from a beneficial effect of treatment with UDCA.

In conclusion, structural reversal of injured hepatocyte organelles is possible, and administration of UDCA greatly alleviates CCl_4-induced alterations of hepatocyte organelles. Nonetheless, these changes should be inspected at different time points during antifibrotic therapy, to identify the association of such structural recovery of different organelles.

COMMENTS

Background

Liver fibrosis and cirrhosis are mostly the results of chronic hepatocyte injury. A drug approved for the management of PBC (UDCA) is the mainstay of therapy of primary biliary cirrhosis (PBC) and has been reported to have antifibrotic activity in the liver. We investigated the ultrastructural changes after UDCA treatment in hepatocytes from experimentally induced liver fibrosis in rats.

Research frontiers

Understanding the changes in hepatocyte organelles during injury and recovery may be advantageous in developing new diagnostic and therapeutic tools.

Innovations and breakthroughs

An ultrastructural scoring system based on the specific findings of individual organelles is to be used for the first time on animal hepatocytes.

Applications

Transmission electron microscopy may be another tool to follow structural alterations in hepatocytes in the disease state.

Terminology

UDCA: A drug approved for the management of PBC.

Peer review

Significant alterations occur in hepatocyte organelles during CCl_4 administration and UDCA improves those changes to a great extent. Transmission electron microscopy may be used to follow morphological changes in the liver during injury and healing.

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RAPID COMMUNICATION

Effect of viral load on T-lymphocyte failure in patients with chronic hepatitis B

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Abstract

AIM: To investigate peripheral T-lymphocyte subpopulation profile and its correlation with hepatitis B virus (HBV) replication in patients with chronic hepatitis B (CHB).

METHODS: Distribution of T-lymphocyte subpopulations in peripheral blood was measured by flow cytometry in 206 CHB patients. HBV markers were detected with ELISA. Serum HBV DNA load was assessed with quantitative real-time polymerase chain reaction (PCR). The relationship between HBV replication and variation in peripheral T-cell subsets was analyzed.

RESULTS: CHB patients had significantly decreased $CD3^+$ and $CD4^+$ cells and $CD4^+/CD8^+$ ratio, and increased $CD8^+$ cells compared with uninfected controls (55.44 ± 12.39 *vs* 71.07 ± 4.76, 30.92 ± 7.48 *vs* 38.94 ± 3.39, 1.01 ± 0.49 *vs* 1.67 ± 0.33, and 34.39 ± 9.22 *vs* 24.02 ± 4.35; $P < 0.001$, respectively). Univariate analysis showed a similar pattern of these parameters was significantly associated with high viral load, presence of serum hepatitis B e antigen (HBeAg) expression, liver disease severity, history of maternal HBV infection, and young age at HBV infection, all with $P < 0.01$. There was a significant linear relationship between viral load and these parameters of T-lymphocyte subpopulations (linear trend test $P < 0.001$). There was a negative correlation between the levels of $CD3^+$ and $CD4^+$ cells and $CD4^+/CD8^+$ ratio and serum level of viral load in CHB patients (r = -0.68, -0.65 and -0.75, all $P < 0.0001$), and a positive correlation between $CD8^+$ cells and viral load (r = 0.70, $P < 0.0001$). There was a significant decreasing trend in $CD3^+$ and $CD4^+$ cells and $CD4^+/CD8^+$ ratio with increasing severity of hepatocyte damage and decreasing age at HBV infection (linear trend test $P < 0.01$). In multiple regression (after adjustment for age at HBV infection, maternal HBV infection status and hepatocyte damage severity) log copies of HBV DNA maintained a highly significant predictive coefficient on T-lymphocyte subpopulations, and was the strongest predictor of variation in $CD3^+$, $CD4^+$, $CD8^+$ cells and $CD4^+/CD8^+$ ratio. However, the effect of HBeAg was not significant.

CONCLUSION: T-lymphocyte failure was significantly associated with viral replication level. The substantial linear dose-response relationship and strong independent predictive effect of viral load on T-lymphocyte subpopulations suggests the possibility of a causal relationship between them, and indicates the importance of viral load in the pathogenesis of T cell hyporesponsiveness in these patients.

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Key words: Hepatitis B virus; Chronic hepatitis B virus infection; Hepatitis B virus DNA; T lymphocyte subpopulation; Immune function

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INTRODUCTION

Hepatitis B virus (HBV) is one of the most prevalent

viral pathogens in humans, with almost a third of the world population having evidence of infection, and about 350 million chronically infected patients[1]. Chronic hepatitis B (CHB) is characterized by inflammatory liver disease of variable severity and is associated with a significantly increased risk of cirrhosis, liver failure and hepatocellular carcinoma[1,2]. Seventy-five percent of patients with CHB are Asian[3]. In China, > 120 million people are chronic carriers of HBV, and 40%-60% catch HBV infection from their mothers[1,2]. For neonates and children younger than 1 year who acquire HBV infection perinatally, the risk of the infection becoming chronic is 90%[1,2].

The pathogenesis of persistent viral infection and hepatitis B is very complex. Both viral factors, as well as the host immune response, have been implicated in the pathogenesis and clinical outcome of HBV infection[4,5]. Apart from direct biological effects of viral variants, there is a growing consensus that the host immune response, especially the virus-specific T cell response, is the key determinant influencing the course of disease and the onset of liver disease[5,6]. Many investigators suggest the chronicity of HBV infection is caused by a deficient cellular immune function, but the mechanism has not been defined[7-9]. For a non-cytopathic virus like HBV to persist, it must either overwhelm or not induce an effective antiviral immune response, or it must be able to evade it. Hepatitis B e antigen (HBeAg) may play an important role in the interaction of the virus with the immune system. Data from transgenic mice indicate neonatal tolerance to HBeAg is a crucial mechanism responsible for the lack of an antiviral immune response following mother to infant transmission[10,11]. Milich *et al*[12] have further demonstrated an immunomodulatory role of HBeAg in antigen presentation and recognition by $CD4^+$ cells.

The relationship between HBV specific T-cell response, HBV viral load and HBeAg expression in CHB is complicated by their close correlation and remains unclear. In China, where vertical perinatal transmission is the main route of transmission, most patients with CHB have become infected in the early years of life. The influence of age at infection and maternal HBV infection status on T-cell immune status and HBV replication is still not settled. The aim of the present study was to evaluate the peripheral blood T lymphocyte subpopulation profile and its correlation with HBV replication.

MATERIALS AND METHODS

Enrollment of study subjects

Two hundred and six consecutive patients with CHB who were admitted to the department of Infectious Diseases of the First Affiliated Hospital of Kunming Medical University, the Third Municipal People's Hospital of Kunming and the Yunnan General Hospital of The Chinese People's Armed Police Forces, between January 2006 and May 2007, were prospectively enrolled into the study. The clinical diagnosis was based on liver function tests, hepatitis virus markers, ultrasonography and histopathology. The diagnosis of all the patients was confirmed according to the criteria for viral hepatitis[13,14]. The following criteria were fulfilled by all patients: (1) steady positivity for hepatitis B surface antigen (HBsAg) in the serum for at least 12 mo, to establish CHB; and (2) exclusion of other concomitant causes of liver disease (hepatitis C, D and HIV infection and alcohol consumption > 60 g/d), relatively rare liver disease (autoimmune hepatitis and metabolic liver disease), and treatment with immunosuppressive therapy or antiviral therapy for HBV infection within the past 12 mo before entry. None of the patients was a drug user, or exposed to hepatotoxin. Those who had liver cirrhosis were also excluded since their long history of treatment and terminal disease state may have complicated the interpretation of the results.

One hundred individuals who were free of HBsAg were identified from those attending the outpatient service for a health check-up; 61 of the participants were male, 39 were female; mean age, 33.24 (SD 10.28) year. These served as the control group for comparison of T-lymphocyte subpopulations with those who had HBV infection.

Serological liver function tests and HBV marker evaluation

Serum alanine aminotransferase (ALT), aspartate transaminase (AST) and total bilirubin were tested with routine automated techniques (upper limit of normal; 40 U/L, 40 U/L and 17.1 μmol/mL, respectively) (AU2700, Japan). HBV markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb, Anti-HBcAb IgM) were measured at a virological laboratory with the use of ELISA (Anthos 2010, Austria). The experimental methods followed those specified within the reagent kit package insert (Sino-American Biotech Co., Ltd, Shanghai, China).

Quantitative measurement of HBV DNA (viremia)

Serum HBV DNA load in patients was assessed with the real-time fluorescent quantitative polymerase chain reaction method (real-time PCR) using a Lightcycler PCR system (FQD-33A, Bioer, Hongzhou, China) with a lower limit of detection of about 1000 viral genome copies/mL. The handling procedures were performed in strict accordance with the reagent kit package insert (Shenzhen PG Biotech Co., Ltd., Shenzhen, China). The primer was provided in the kit, the reaction volume was 40 μL, and the reaction conditions were 37℃ for 5 min, 94℃ for 1 min, then 40 cycles at 95℃ for 5 s and 60℃ for 30 s. Results were considered abnormal when HBV DNA was > 1000 copies/mL.

Peripheral blood T lymphocyte subsets measurement

Blood samples were collected in heparinized vacutainer tubes. Samples were analyzed with a Muti-Q-Prep processor (Coulter, USA) and thereafter Epics-XL flow cytometry (FCM) (Coulter). Lymphocytes were analyzed using a gate set on forward scatter versus side scatter. Anti-human monoclonal antibodies CD3-PE-CY5/CD4-FITC/CD8-PE were purchased from Immunotech (USA). For each sample, detection was carried out using CELLQuest software (Coulter). The results were expressed as the percentages of $CD3^+$, $CD4^+$ and $CD8^+$ cells found to be positive for the marker antigen in the total T cell

Table 1 Characteristics of patients with chronic hepatitis B

Characteristics	All patients	Various courses of chronic hepatitis B			
	(*n* = 206)	Mild-CHB (*n* = 101)	Moderate-CHB (*n* = 54)	Severe-CHB (*n* = 51)	*P* value
Sex (male/female)	143/63	68/33	36/18	39/12	> 0.05[2]
Mean age (yr)[1]	29.61 ± 9.38	29.06 ± 9.88	29.72 ± 9.16	30.59 ± 8.67	> 0.05[3]
Patients with MH, *n* (%)	104 (50.5)	45 (44.6)	23 (42.6)	36 (70.6)	< 0.01[2]
Age at HBV-infection (yr), *n* (%)					< 0.01[2]
< 8	86 (41.7)	34 (33.7)	22 (40.7)	30 (58.8)	
8-20	71 (34.5)	39 (38.6)	22 (40.7)	10 (19.6)	
> 20	45 (21.8)	28 (27.7)	8 (14.8)	9 (3.9)	
unknown	4 (1.9)	0	2 (3.7)	2 (3.9)	
Serum ALT (IU/L)[1]	163.75 ± 140.41	69.91 ± 17.36	152.04 ± 19.62	379.65 ± 186.44	< 0.001[3]
Serum AST (IU/L)[1]	116.69 ± 107.97	52.61 ± 14.13	96.26 ± 21.27	265.25 ± 125.12	< 0.001[3]
Serum total bilirubin (μmol/mL)[1]	20.79 ± 14.02	15.18 ± 2.42	19.20 ± 4.51	33.59 ± 23.22	< 0.001[3]
HBV DNA positive, *n* (%)	149 (72.3)	51 (50.5)	47 (87.0)	51 (100.0)	< 0.001[2]
Serum HBV DNA (copies/mL), *n* (%)					< 0.001[2]
$\leqslant 1.0 \times 10^3$	57 (27.7)	50 (49.5)	7 (13.0)	0	
$> 1.0 \times 10^3$-1.0×10^5	32 (15.5)	14 (13.9)	18 (33.3)	0	
$> 1.0 \times 10^5$-1.0×10^7	31 (15.0)	11 (10.9)	11 (20.4)	9 (17.6)	
$> 1.0 \times 10^7$	86 (41.7)	26 (25.7)	18 (33.3)	42 (82.4)	
HBV DNA load (Log, copies/mL)[1]	5.84 ± 2.22	4.77 ± 2.11	5.91 ± 1.83	7.91 ± 1.17	< 0.001[3]
HBeAg positive, *n* (%)	96 (46.6)	25 (24.8)	31 (57.4)	40 (78.4)	< 0.001[2]

HBV: Hepatitis B virus; CHB: Chronic hepatitis B; MH: Maternal HBV-infection status. [1]Date are expressed as mean ± SD; [2]Chi-square test *P*-value; [3]ANOVA *P*-value.

population. The handling procedures were performed in strict accordance with the manufacturer's instructions.

Maternal HBV infection status (MH)

MH was confirmed according to the maternal presence of serum HBV markers and/or HBV DNA, documented on at least two occasions, at least 3 mo apart; or documented maternal death from HBV-related liver diseases such as CHB, HBV-related liver cirrhosis and/or hepatocellular carcinoma.

Age at HBV infection

In the past three decades in China, all children have been obliged to be tested for HBV markers when they first go to kindergarten and elementary school. Subsequent obligatory tests are carried out when they apply for university or a job. The results of these tests were obtained from medical records and interviews. We classified the age of the first positive test as < 8 years, 8-20 years and > 20 years old.

Statistical analysis

Initial sample size calculation came up with 50 subjects positive for HBV DNA positive and the same number negative. This provided the study with a statistical power of 80% at the 0.025 level of significance to detect a difference in T-cell variation values of 33 *versus* 38. However, to cover the problem of being potentially confounded by other variables, and to have enough subjects for stratifying levels of HBV DNA load to examine a dose-response relationship, 206 CHB patients and 100 controls were recruited.

Descriptive statistics were used to examine the age, gender, serum HBV load, HBeAg status, ALT, AST, total bilirubin, age at HBV infection and maternal HBV infection status. The levels of T-lymphocyte subpopulation in normal individuals (HBsAg-negative) were summarized as means ± SD, to serve as a control reference. Effects of various independent demographic, clinical and serological variables on T-cell profile were analyzed only among HBsAg-positive individuals. In univariate analysis, breakdown of these profiles by individual independent variables was carried out. An independent *t* test was done for two-level independent variables and one-way ANOVA for more than two-level variables. The relationship between HBV replication and peripheral T-lymphocyte subpopulation was analyzed by correlation analysis and ANOVA linear trend test. Finally, a multiple linear regression model was employed for multivariate analysis to assess the independent effects of variables on peripheral blood T lymphocytes. Variables yielding $P \leqslant 0.2$ in univariate analysis were included in the multivariate analysis, and the models were refined by backward elimination, guided by a change in log likelihood of successive models. A final $P < 0.05$ was considered statistically significant. Computations were carried out with the aid of R software version 2.5.1[15].

RESULTS

Demographic characteristics and clinical features of CHB patients

The demographic, virological, serological and clinical characteristics of the patients are summarized in Table 1.

Over two-fifths of the CHB patients acquired the infection before the age of 8 year. Almost three-quarters had detectable levels of HBV DNA. Among these, the majority (57.7%) had > 10^7 copies/mL. Around half of the patients' mothers were HBV-positive. A little less than half were HBeAg-positive (46.6%). Among various courses of CHB, all severe-CHB patients had detectable levels of HBV DNA; the majority of severe-CHB patients

Table 2 Peripheral T-cell subsets in normal control and CHB patients broken down by various factors (mean ± SD)

Groups	*n*	CD3$^+$	CD4$^+$	CD8$^+$	CD4$^+$/CD8$^+$ ratio
HBV status[b]					
Negative (normal control)	100	71.07 ± 4.76	38.94 ± 3.39	24.02 ± 4.35	1.67 ± 0.33
Positive	206	55.44 ± 12.39	30.92 ± 7.48	34.39 ± 9.22	1.01 ± 0.49
Maternal HBV-infection status[b]					
Negative	102	60.36 ± 10.68	34.92 ± 6.52	30.43 ± 7.76	1.25 ± 0.49
Positive	104	50.60 ± 12.09	26.99 ± 6.18	38.29 ± 8.91	0.77 ± 0.34
Age at HBV-infection (yr)[d]					
< 8	86	49.83 ± 11.64	25.74 ± 5.16	39.11 ± 8.68	0.70 ± 0.24
8-20	71	59.28 ± 11.47	34.15 ± 6.31	31.49 ± 7.52	1.18 ± 0.47
> 20	45	60.29 ± 11.13	35.58 ± 7.27	29.68 ± 8.80	1.33 ± 0.54
Unknown	4	53.20 ± 12.45	32.63 ± 4.43	37.78 ± 5.54	0.88 ± 0.19
Liver diseases status[d]					
Mild-CHB	101	60.38 ± 11.51	33.57 ± 8.36	30.29 ± 8.91	1.23 ± 0.54
Moderate-CHB	54	56.69 ± 10.27	30.53 ± 4.79	35.26 ± 7.72	0.93 ± 0.31
Severe-CHB	51	44.32 ± 8.71	26.08 ± 5.19	41.63 ± 6.18	0.65 ± 0.19
HBV DNA load (copies/mL)[f]					
$\leqslant 1.0 \times 10^3$	57	66.13 ± 7.38	37.92 ± 5.94	25.18 ± 5.39	1.58 ± 0.43
$> 1.0 \times 10^3$-1.0×10^5	32	60.93 ± 8.02	32.73 ± 6.42	33.18 ± 5.36	1.03 ± 0.33
$> 1.0 \times 10^5$-1.0×10^7	31	54.28 ± 12.91	29.63 ± 7.07	35.17 ± 8.18	0.88 ± 0.25
$> 1.0 \times 10^7$	86	46.72 ± 9.31	26.07 ± 4.52	40.69 ± 7.35	0.67 ± 0.22
HBeAg status[b]					
Negative	110	61.09 ± 10.67	33.99 ± 7.48	30.17 ± 8.04	1.24 ± 0.52
Positive	96	48.99 ± 11.06	27.39 ± 5.75	39.24 ± 8.05	0.73 ± 0.25

[b]$P < 0.001$ for all comparisons of +ve *vs* -ve for each measure and each T-cell parameter; [d]$P < 0.01$, [f]$P < 0.001$ for ANOVA Linear trend test.

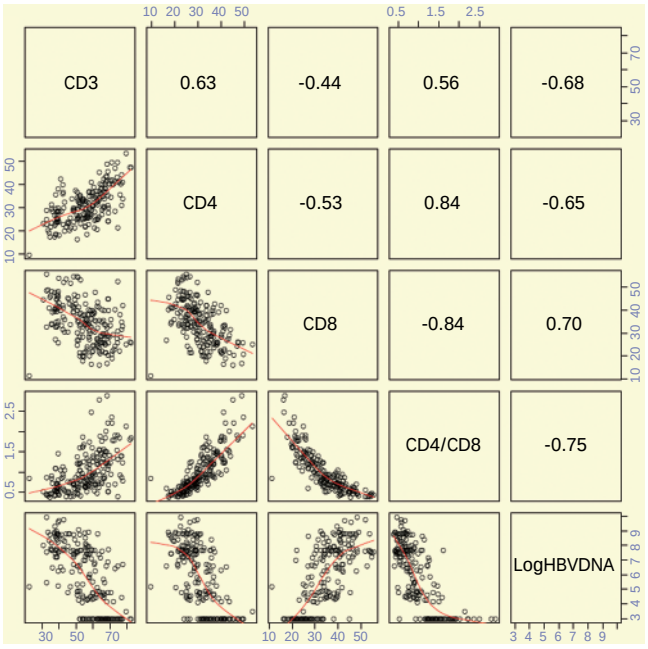


Figure 1 Correlation between peripheral T-cell subsets and serum level of HBV viral load.

had higher viral load (82.4%), higher titer of HBV DNA in serum, higher serum positivity of HBeAg (78.4%), higher maternal HBV infection status (70.6%) and lower age at HBV-infection than mild-CHB and moderate-CHB patients (all $P < 0.01$). The severity of liver diseases was significantly associated with HBV replication status, including higher viral load and HBeAg expression, all with $P < 0.001$ (Table 1).

Over two-thirds of the CHB patients with MH acquired the infection before the age of 8 year (79.8%), whereas age of HBV infection of > 20 years was more common in non-MH patients. Higher positivity of serum HBV DNA and of HBeAg were observed in MH patients than in non-MH individuals (92.3% *vs* 52.0%, OR = 10.96, 95% CI 4.7-28.85; 56.7% *vs* 36.3%, OR = 2.29, 95% CI 1.27-4.19; respectively). The percentage of cases with higher-viral-load viremia (> 10^7 copies/mL) was higher in MH patients than that in the non-MH group (57.7% *vs* 25.5%, OR = 3.96, 95% CI 2.12-7.54). A greater proportion of non-MH individuals had an undetectable-viral-load viremia (< 10^3 copies/mL) compared with MH individuals (48.0% *vs* 7.7%, OR = 10.96, 95% CI 4.7-28.85). A higher double-positivity of serum HBV DNA and HBeAg existed in MH individuals, significantly higher than in non-MH patients (56.7% *vs* 36.3%, OR = 2.29, 95% CI 1.27-4.19).

Peripheral T-lymphocyte subpopulation composition in CHB patients

CHB patients had significantly decreased total CD3$^+$ and CD4$^+$ subpopulations and CD4$^+$/CD8$^+$ ratio, and increased CD8$^+$ subsets compared with uninfected controls, all with $P < 0.001$. Univariate analysis showed that T-cell failure was significantly associated with higher viral load, serum HBeAg expression, severity of liver disease, history of maternal HBV infection, and lower age at HBV infection (Table 2). Linear dose-response relationship between the level of T-lymphocyte subpopulation and copies of HBV DNA was also highly significant (linear trend test $P < 0.001$). A negative correlation existed between the levels of CD3$^+$ and CD4$^+$ cells and CD4$^+$/CD8$^+$ ratio and serum HBV viral load, whereas a positive correlation existed between the level of CD8$^+$ cells and viral load, all with $P < 0.0001$. Correlation between T lymphocytes and viral load is shown in Figures 1 and 2.

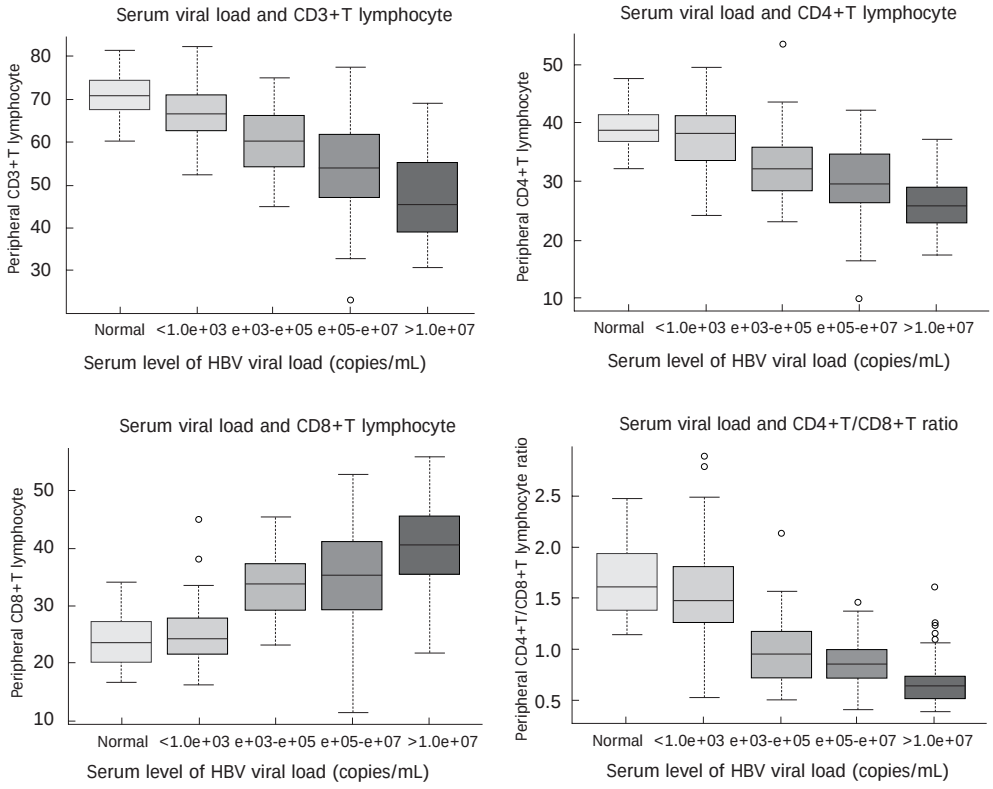


Figure 2 Peripheral T-lymphocyte subpopulations by serum HBV viral load level. On the figure, the marks "<1.0e+03", "e+03-e+05", "e+05-e+07" and ">1.0e+07" denote "< 10^3", "10^3-10^5", "10^5-10^7" and "> 10^7", respectively.

Table 3 Multiple linear regression predicting peripheral blood T lymphocyte subpopulations (n = 206)

	$CD3^+$ T lymphocyte			$CD4^+$ T lymphocyte			$CD8^+$ T lymphocyte			$CD4^+/CD8^+$ ratio		
	β	SE	*P* value	β	SE	*P* value	β	SE	*P* value	β	SE	*P* value
Intercept	69.39	3.51		35.6	2.07		23.21	2.56		1.55	0.12	
Serum HBV load (Log, copies/mL)[1]	-2.58	0.45	< 0.0001	-1.31	0.26	< 0.0001	2.05	0.33	< 0.0001	-0.11	0.02	< 0.0001
HBeAg[2]	2.37	1.65	0.15	0.71	0.98	0.47	-1.16	1.21	0.34	0.06	0.06	0.32
Liver diseases status[3]			< 0.01			0.63			0.04			0.04
Moderate-CHB	-0.36	1.58		-1.15	0.93		2.34	1.15		-0.15	0.05	
Severe-CHB	-6.14	1.88		-1.65	1.11		3.52	1.37		-0.14	0.06	
Age at HBV-infection (yr)[4]			0.31			< 0.001			0.06			< 0.001
8-20	-0.06	2.09		4.1	1.24		-0.72	1.52		0.13	0.07	
> 20	-1.72	2.55		4.03	1.51		-0.16	1.86		0.15	0.09	
Unknown	1.93	4.94		6.17	2.92		-0.52	3.61		0.14	0.17	
Maternal HBV-infection status[5]	3.61	2.05	0.08	1.78	1.21	0.14	-2.76	1.49	0.07	0.14	0.07	0.05

β: Coefficients from the model. [1]Continuous variable; [2]Reference group: HBeAg-negative; [3]Reference group: mild-CHB; [4]Reference group: < 8 yr old age at HBV infection; [5]Reference group: With maternal HBV-infection status.

Furthermore, there was a significant decreasing trend of $CD3^+$ and $CD4^+$ cells and $CD4^+/CD8^+$ ratio with increasing hepatocytic damage; this was inverse for $CD8^+$ cells. A similar pattern was also seen among age at HBV infection, all with a linear trend test *P* value < 0.01.

Linear regression predicting peripheral blood T-lymphocyte subpopulation from relevant parameters

In Table 3, linear regression models are separately summarized for $CD3^+$, $CD4^+$ and $CD8^+$ cells and $CD4^+/CD8^+$ ratio, which are the dependent variables. After adjustment for all independent variables listed in the table, serum HBV viral load was the key predictor for T-cell profile. The severity of liver disease reduced the number of $CD3^+$ T lymphocytes, increased the number of $CD8^+$ T cells, and decreased the $CD4^+/CD8^+$ ratio. Those who had infection at a young age had a lower $CD4^+$ T cell count and $CD4^+/CD8^+$ ratio than those who acquired infection later in life. Maternal infection history and serum HBeAg expression had no independent effect on T-lymphocyte profile.

DISCUSSION

This study demonstrated disorder of cellular immune function in CHB patients. The level of T-cell dysfunction had a linear dose-response relationship with the load of HBV DNA. Furthermore, the study also illustrated that the strong independent effect of HBV viral load seemed to eliminate and/or weaken the effects of liver disease severity, maternal carrier status, early age of infection and HBeAg positivity on the impairment of T-cell function.

Our findings indicate that CHB patients have T-cell failure. The same finding has also been demonstrated previously, namely, that the chronicity of HBV infection is caused by a deficiency in cellular immune function[16-20], and hepatocyte damage is mainly caused by immunological injury[21-29]. However, the mechanism has not been defined[5]. Apart from direct biological effects of viral variants, there is a growing consensus that the host immune response, especially the virus-specific T-cell response, is the key determinant influencing the course of disease and the onset of liver disease[5,6,30]. The significant decrease in total T lymphocyte ($CD3^+$ T) revealed that there is a lack of immunologically competent cells involved in cellular immunoreactivity against HBV infection. A lack of $CD4^+$ T cells can impair $CD8^+$ T-cell activity and antibody production[31], while the inability to mount a virus-specific $CD8^+$ T-cell response results in a level of circulating virus that cannot be cleared by antibodies alone[32-34]. Activation-induced cell death (AICD) is related to a decrease in lymphocytes and functional defects. This phenomenon can cause decreased immune clearance. This may be an important reason for persistent infection with HBV. AICD in peripheral blood T lymphocytes in CHB has been demonstrated previously[35,36]. Thus, AICD is considered an important modulator in down-regulating the "burst" of responding T cells in patients with CHB[36].

Our results revealed T-cell failure was significantly associated with viral replication level. The substantial linear dose-response relationship and strong independent predictive effect of HBV DNA, but not other variables, on T-lymphocyte subpopulations suggests the possibility of a causal relationship between them. However, the cross-sectional nature of our data did not allow us to identify the temporal direction of the causal relationship between these two variables. Mizukoshi *et al*[37] have suggested antiviral therapy of persistently infected patients appears to increase the frequency of HBV-specific $CD4^+$ T-cell responses during the first year of treatment. Boni *et al*[38,39] have reported antiviral treatment can overcome $CD8^+$ T-cell hyporesponsiveness in subjects with CHB, which suggests the T cells are present but suppressed. It has been reported by Pham *et al*[40] in 21 CHB patients that the ratio of $CD4^+/CD8^+$ liver-derived lymphocytes, but not of peripheral blood lymphocytes, appears to be related to the level of HBV replication, which reveals a positive correlation with viral load. The evidence that an efficient antiviral T-cell response can be restored by antiviral monotherapy in CHB, concurrently with reduction of viremia, indicates the importance of viral load in the pathogenesis of T-cell hyporesponsiveness.

The strong independent effect of viral load on T-cell impairment and viral factors (viral variants) might explain the disappearance of the effect of other variables in multivariate analysis. Among our patients, the majority were characterized by young age at first HBV infection, maternal carrier status, and high serum viral load, especially in severe CHB patients. In addition to HBV DNA, HBeAg is also a serological marker for viral replication, which plays a crucial role in chronicity of HBV infection and high viral load, by inducing immunological tolerance to HBV in the fetus. The tolerance-inducing effect of HBeAg has been well characterized in mice[41-43] and likely contributes to the low level of core-specific T-cell responses present in HBeAg-positive CHB patients[4,5]. Clinical evidence supports the tolerogenic effect of HBeAg[5,44]. Also, viral mutations that abrogate or antagonize antigen recognition by virus-specific T cells have been reported in patients with CHB[45,46]. Although the results from univariate analysis in our study showed T-cell dysfunction was significantly related to HBeAg, the association disappeared in multivariate analysis. One possible reason is that some of the subjects were infected with pre-C stop codon mutation virus (pre-C/C mutant), which resulted in a loss of HBeAg. In these patients, therefore, viral replication may have persisted, despite elimination of HBeAg and seroconversion to anti-HBe. While the loss of HBeAg appears irrelevant to the biology of the virus, it may play an important role in the interaction of the virus with the immune system. This may weaken the independent association between HBeAg and T-cell failure, so that the sample size in our study could not detect this magnitude of association. Moreover, those who had a history of maternal carriage usually acquired infection at a younger age, and a higher HBV viral load was detected in the majority of those who had infection at a younger age. In the same way, those who had severe liver damage were usually positive for maternal HBV carrier status and acquired infection in early life, thus a high viral load was measured in these patients. This phenomenon suggests that infection from the mother and/or at younger age predisposes to tolerance to HBV infection and thus, higher viral load.

Our study clearly showed the severity of the liver disease was significantly associated with functional disorder of T-lymphocytes, and the effect was independent of viral load. Hepatocyte damage may also be correlated directly with T-cell failure, rather than through the load copies of viral replication. Previous studies have suggested that hepatocyte damage is mainly caused by immunological injury[9,21-30]. HBV is a typical non-cytopathic virus that can induce tissue damage of variable severity by stimulating a protective immune response that can simultaneously cause damage and protection, by killing an intracellular virus through the destruction of virus-infected cells[5]. Therefore, immune elimination of infected cells can lead to the termination of infection when it is efficient, or to a persistent necroinflammatory disease when it is not[47]. Destruction of infected cells, however, is not the only mechanism implicated in the elimination of intracellular virus, as demonstrated by studies carried out in animal models of HBV infection and in human hepatitis B, which demonstrate the importance of cytokine-mediated, non-cytolytic mechanisms of anti-viral protection[20-31].

The strength of this study lies in the large sample size and the measurements of T-lymphocyte subpopulations using modern advanced FCM technology and viral load with quantitative real-time PCR. A limitation of this study is that the specificity of T-lymphocyte subpopulations and liver-derived T-lymphocytes were not explored concurrently. Although a strong relationship between T-lymphocyte subpopulations and viral load was illustrated, further studies are needed to confirm the causal relationship between them.

Our results, which suggest high viral load contributes to functional impairment of T cells in CHB patients, have practical implications for understanding the pathogenesis and control of persistent viral infection and diseases progression and prognosis. This is because patients with CHB are at risk of persistent viral infection that leads to liver failure, cirrhosis and even hepatocellular carcinoma[1,2]. We should take into account effective intervention strategies such as anti-viral and/or immunotherapy to prevent progression and long-term consequences. Inhibition of viral replication with agents such as lamivudine may enhance the likelihood that therapeutic stimulation of the T-cell response will induce HBV antigen seroconversion, ultimately leading to recovery from disease. Further clinical studies are needed to explore this possibility in persistent HBV-infected patients.

In conclusion, we found a strong, independent predictive effect of viral load on T-lymphocyte subpopulations, which suggests a causal relationship between viral load and T-cell failure. T-cell dysfunction might contribute to viral persistence. HBV establishes persistent infection mainly by vertical transmission from HBV-infected mothers to neonates, and the immunomodulatory effects of HBeAg might play an important role in this setting. High viral load may be one important factor that contributes to T-lymphocyte failure, and is more important than HBeAg in this regard. Clearly, additional studies are required to better understand the complex host-virus interactions that determine the persistence and outcome of HBV infection.

COMMENTS

Background

HBV infection is a global public health problem. Infection with hepatitis B virus (HBV) leads to a wide spectrum of clinical presentations ranging from an asymptomatic carrier state to self-limited acute or fulminant hepatitis to chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma. But the pathogenesis of persistent viral infection and hepatitis B is very complex and has not been clarified until now. Generally, it is not HBV itself that damages hepatocytes directly, but the results of function disorder of cell-mediated immunity. The outcome of HBV infection would depend upon the balance between development of immunity (leading to virus elimination) and tolerance (leading to chronic viral persistence).

Research frontiers

Outcome of infection and the pathogenesis of liver disease are determined by virus and host factors, which have been difficult to fully elucidate because the host range of HBV is limited to man and chimpanzees. The pathogenesis of liver disease and interaction between virus and host remain the research hotspots in this field.

Innovations and breakthroughs

The pathogenesis and correlation of functional disorder of cellular immune and viral replication level remains unknown. In our study, peripheral T-lymphocyte subpopulation of chronic hepatitis B (CHB) patients in large sample size were measured using advanced flow cytometry technology and viral load with quantitative real-time polymerase chain reaction (PCR) method. The results suggest T-lymphocyte failure was significantly associated with viral replication level. The substantial linear dose-response relationship and strong independent predictive effect of viral load on T-lymphocyte subpopulations suggests close proximity of the causal pathway between them, and indicates the importance of viral load in the pathogenesis of T cell hyporesponsiveness in these patients.

Applications

The results, which suggest high viral load contributes to functional impairment of T-cell in CHB patients, have practical implications because the understanding of the immune response upon HBV infection is useful in developing appropriate therapeutic strategies for controlling viral hepatitis and disease progression, as well as for improving current knowledge regarding persistent HBV infection prognosis. In addition, it will be possible to predict the variation of T-lymphocyte subpopulations in peripheral blood in the future by measuring serum viral load level in chronic HBV-infected patients.

Terminology

$CD4^+$ T cells, classically referred to as helper T cells that are required for the efficient development of effector cytotoxic/suppressor $CD8^+$ T-cell and B-cell antibody production, play an important role in HBV infection by secretion of Th1 cytokines that down-regulate HBV replication, and by promoting $CD8^+$ T-cell and B-cell responses. $CD8^+$ T cells go on to clear HBV-infected hepatocytes through cytolytic and non-cytolytic mechanisms, reducing the levels of circulating virus, while B-cell antibody production neutralizes free viral particles and can prevent (re)infection. $CD3^+$, $CD4^+$ and $CD8^+$ cells are major function subgroups of T cells and play an important role in response to HBV infection, which can reflect the situations of cellular immune function and immunoregulation and are usually regarded as a valuable index to forecast the changes of patients' immunity.

Peer review

The authors demonstrated the correlation between T-lymphocyte subpopulation profile and HBV replication level in Chinese patients. Their findings are of some importance.

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RAPID COMMUNICATION

Comparative analysis of intestinal microbial community diversity between healthy and orally infected ducklings with *Salmonella enteritidis* by ERIC-PCR

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Abstract

AIM: To analyze the difference of intestinal microbial community diversity between healthy and (*S. enteritidis*) orally infected ducklings.

METHODS: Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR was applied to analyze the intestinal microbial community diversity and dynamic change including duodenum, jejunum, ileum, cecum and rectum from healthy ducklings and 7-day-old ducklings after oral infection with *S. enteritidis* at different time points.

RESULTS: The intestinal microbial community of the control healthy ducklings was steady and the ERIC-PCR band numbers of the control healthy ducklings were the least with rectum and were the most with caecum. ERIC-PCR bands of orally inoculated ducklings did not obviously change until 24 h after inoculation (p.i.). The numbers of the ERIC-PCR bands gradually decreased from 24 h to 72 h p.i., and then, with the development of disease, the band numbers gradually increased until 6 d p.i. The prominent bacteria changed because of *S. enteritidis* infection and the DNAstar of staple of ERIC-PCR showed that aerobe and facultative aerobe (*Escherichia coli*, *Shigella*, *Salmonella*) became preponderant bacilli in the intestine of orally infected ducklings with SE.

CONCLUSION: This study has provided significant data to clarify the intestinal microbial community diversity and dynamic change of healthy and *S. enteritidis* orally infected ducklings, and valuable insight into the pathogenesis of *S. enteritidis* infection in both human and animals.

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Key words: ERIC-PCR; *Salmonella enteritidis*; *Salmonella enteritidis* infected ducklings; Intestinal microbial communities

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INTRODUCTION

Salmonella enteritidis (*S. enteritidis*) remains one of the main causes of food-borne illness and as such is considered to be the most important pandemic zoonosis produced under natural conditions[1-3]. It is estimated that the worldwide incidence is more than a thousand million cases, causing three million deaths each year. *S. enteritidis* can through the food production chain pass to humans, therefore

undercooked or raw eggs and poultry meat are of a high risk for humans[4,5]. Infections caused by Salmonella bacteria belong to the most common zoonotic diseases worldwide[6]. Outbreaks of human salmonellosis caused by *S. enteritidis* have dramatically increased throughout the world since the mid-to-late 1980s and have become an important problem for the poultry industry and public health.

Intestinal microflora plays an important role in digestive function, immunity, and disease resistance. It becomes clear that intestinal resident and in-transit micro-organisms play an essential role in the homeostasis of local and systemic immunity[7,8]. A large number of microbial species exist in gastrointestinal tract. However, traditional culture-based techniques are inappropriate in studying the structure-function relationship of microbial communities due to their highly selective, laborious and time-consuming nature. Only a relatively small fraction of population members in a complex natural community, such as gastrointestinal system, can be recovered with culture-based techniques[9]. ERIC-PCR method developed in recent years can simultaneously compare sequence-based structural features of large numbers of community samples[10-14]. In this study, to understand the changes of intestinal microbial communities of *S. enteritidis*-infected ducklings, ERIC-PCR method was used to fingerprint the kinetics of microbial community of fecal samples of orally infected ducklings with *S. enteritidis*, which will provide valuable insight into the pathogenesis of *S. enteritidis* infection.

MATERIALS AND METHODS

Bacterial strain

S. enteritidis (Duck, No.MY1) was isolated and maintained by Avian Disease Research Center, College of Veterinary Medicine of Sichuan Agricultural University.

Ducklings

The study was conducted with 68 7-day-old white Peking ducklings (the serum and feces samples of all experimental ducklings were tested and found to be *S. enteritidis* negative by PCR methods). All these ducklings were maintained in isolation units in a biosecure animal building and fed a commercial ducklings diet ad libitum. Two groups of ducklings were included in this study. Group 1 was used to test the dynamic change of intestinal microbial community diversity and 48 ducklings in this group were randomly divided into part A (24 orally inoculated ducklings) and part B (24 control healthy ducklings). Ducklings in part A were orally infected with 0.2 mL *S. enteritidis* culture solution (4×10^9 CFU/mL) and ducklings in part B were simultaneously administrated with 0.2 mL isotonic Na chloride. The clinical signs and pathological lesions of infected ducklings were monitored until 21 d after inoculation (p.i.) and the mortality was recorded in Group 2. In this group, 20 ducklings were averagely divided into two parts (C and D) at random. Ten ducklings in part C were orally infected with 0.2 mL *S. enteritidis* culture solution (4×10^9 CFU/mL) and 10 ducklings in part D took simultaneously 0.2 mL isotonic Na chloride.

Collection of samples

Ducklings in Group 1 were killed and sampled at 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 36 h, 48 h, 72 h, 6 d, 9 d p.i.. At each of the 12 sampling time points between 30 min and 9 d p.i., two ducklings of each part in Group 1 were chosen randomly for sampling. Five samples were collected from each duckling, including contents from 3 cm-long duodenum, jejunum, ileum, caecum and rectum tissues. Fresh samples were collected, put into 80% glycerol and stored at -70℃ before DNA extraction.

Total DNA extraction and purification

DNA extraction procedure modified from Wei G *et al*[15] was used to prepare the DNA templates for PCR reactions to ensure that stable, representative and reproducible fingerprints were obtained. The sample above was suspended in 15 mL sterile 0.05 mol/L phosphate buffered saline (PBS) (1 L contained 1.48 g $NaH_2PO_4.2H_2O$, 14.45 g NaH_2PO_4, 4.0 g NaCl; pH 7.4) followed by vortexing for 15 min in an 50-mL tube. The suspension was centrifuged at 600 × *g* for 5 min and transferred to a new tube. This was repeated three times to remove coarse particles. The cells in the supernatant were collected and washed once by centrifuge at 9000 × *g* for 3 min followed by resuspension in 15 mL 0.05 mol/L PBS. The cell pellets were washed three times by centrifuge at 9000 × *g* for 5 min in 5 mL acetone precooling at -20℃. The washed cell pellets were collected in a new 1.5 mL tube and resuspended in 200 μL solution I (50 mmol/L Tris-base, 50 mmol/L Na_2EDTA, pH 7.8), the suspension was mixed with 40 μL lysozyme solution (50 mg/mL), water-bathed for 30 min at 37℃ with gentle mixing by inverting the tube every 10 min, and then was combined with 500 μL solution Ⅱ (200 mmol/L NaCl, 100 mmol/L Tris-base, 50 mmol/L Na_2EDTA, 2.0% SDS, 1.0% Triton X-100) and 12.5 μL Proteinase K, water-bathed for 12 h at 37℃. RNA was digested by adding RNase (0.80 g/L) at 37℃ for 20 min. DNA was then purified by sequential extraction with Tris-equilibrated phenol, phenol and chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1) followed by precipitation with two volumes of ethanol. DNA was collected by centrifugation, air dried and dissolved in 40 μL sterile TE buffer.

The total DNA samples prepared with the above protocol were characterized with agarose gel electrophoresis for integrity and size. The DNA was adjusted to 40 ng/μL and stored at -20℃ before used as templates for PCR. Two μL DNA sample was added to 23 μL PCR mixtures for each PCR reaction.

DNA fingerprinting

Community fingerprints were obtained for intestinal microflora using total DNA as templates for ERIC-PCR. The sequence of the ERIC primers was based on work by Versalovic *et al*[16], E1 (ERIC1): 5'-ATGTAA GCTCCTGGGGATTCAC-3', E2 (ERIC2): 5'-AAGT AAGTGACTGGGGTGAGCG-3'. Each 23 μL PCR reaction mixture contained 25 pmol of each primer, 5 mmol/L each deoxynucleoside triphosphate (dNTP) and 2.5 U of Taq DNA polymerase (Promega, USA) in the manufacturer's provided buffer. Two microliters of

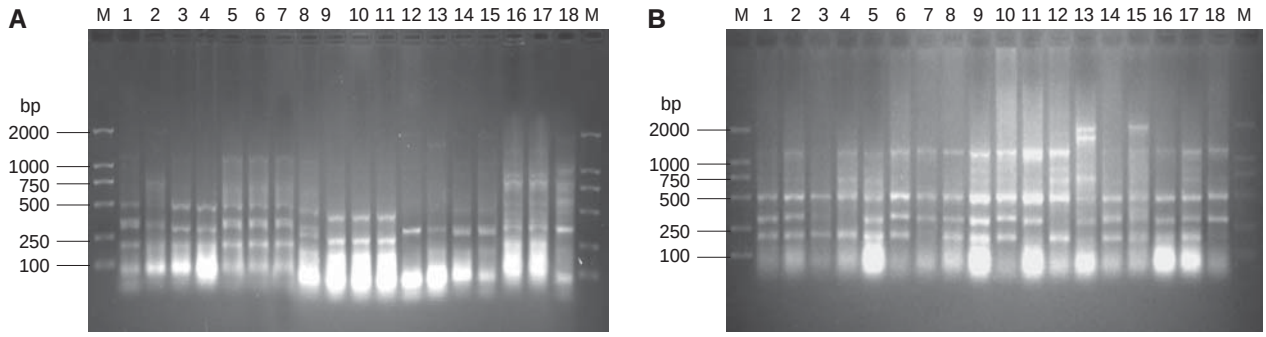


Figure 1 ERIC-PCR results of healthy and orally infected ducklings at different time points. (**A**) M: DL-2000; 1: 6 d duodenum; 2: 72 h duodenum; 3: 24 h duodenum; 4: 1 h duodenum; 5: 6 d ileum; 6: 72 h ileum; 7: 24 h ileum; 8: 1 h ileum; 9: 6 d jejunum; 10: 72 h jejunum; 11: 24 h jejunum; 12: 1 h jejunum; 13: 6 d rectum; 14: 24 h rectum; 15: 1 h rectum; 16: 6 d caecum; 17: 24 h caecum; 18: 1 h caecum. (**B**) M: DL-2000; 1: 1 h duodenum; 2: 24 h duodenum; 3: 72 h duodenum; 4: 6 d duodenum; 5: 1 h jejunum; 6: 24 h jejunum; 7: 72 h jejunum; 8: 6 d jejunum; 9: 1 h ileum; 10: 24 h ileum; 11: 72 h ileum; 12: 6 d ileum; 13: 1 h caecum; 14: 24 h caecum; 15: 6 d caecum; 16: 1 h rectum; 17: 24 h rectum; 18: 6 d rectum.

total fecal DNA (about 80 ng) was added to reach the final 25 μL PCR reaction volume. PCR amplifications were performed in an automated thermocycler (Hybaid, USA) using the following program: 7 min at 95℃; 30 cycles of denaturation at 95℃ for 30 s, annealing at 50℃ for 1 min and extension at 65℃ for 8 min; followed by a final extension at 65℃ for 16 min[14]. The concentrations of the PCR products were determined by DNA Quant 200 fluorometer (Pharmacia, USA) and resolved in 1.5% agarose gel electrophoresis with 300-400 ng total PCR products loaded in each lane. The gels were stained with ethidium bromide and photographed with Bio-Rad, VersaDoc Model 2000.

Cloning and sequencing of DNA fragments from major bands[17]

Gel slices containing individual major DNA bands were excised and transferred into sterile 1.5 mL Eppendorf tubes and purified by UltraClean 15 DNA Purification Kit (MO BIO, USA), according to the manufacturer's instructions. The purified DNA was dissolved in 30 μL sterile water and the concentration was determined by DyNA Quant 200 fluorometer (Pharmacia). The purified fragments were ligated with vector pGEM-T Easy (Promega) and transformed into Escherichia coli DH5α. The transformants were plated on Luria-Bertani (LB) plates containing ampicillin and X-Gal/IPTG. The plasmids were extracted using the alkaline lysis method. For each part of experimental group, two clones were selected for sequencing (TaKaRa, Beijing). The sequences were analyzed with the BLAST program at the NCBI website (http://www.ncbi.nlm.nih.gov/blast).

RESULTS

Clinical observation of ducklings orally infected with SE

Similar clinical signs and gross findings were observed in orally infected ducklings with *S. enteritidis*. Typical clinical signs including inappetence, depression, lameness, fervescence, and white diarrhea were observed in all infected ducklings from around 3 d p.i., and some infected ducklings had nervous symptom and tremor of head-neck. Two inoculated ducklings died as a result of *S. enteritidis* infection between 48 h and 60 h p.i. Necropsy revealed similar lesions in all dead ducklings, including hemorrhages of the heart, hydropericardium, exudative fibrin on the surface of the visceral pericardium, hyperaemia and haemorhage of liver. Hyperaemia and haemorhage of intestinal tract was also observed. As a result of *S. enteritidis* infection, 8 of 10 infected ducklings in part C of Group 2 survived and exhibited no clinical signs except growing slowly and survived throughout the infection, so the mortality of the seven-day-ducklings infected with MY1 strain of *S. enteritidis* was 20%.

The control healthy ducklings in part D of Group 2 had no specific clinical signs and gross findings.

Dynamic monitoring of intestinal microflora in the control healthy ducklings

The ERIC-PCR fingerprints analysis showed that the intestinal microbial community of the control healthy ducklings in part B of Group 1 was steady. The numbers of the band formed by ERIC-PCR with rectum were the least and were the most with caecum. However, the band numbers of duodenum, ileum and jejunum between that of rectum and caecum (Figure 1A) had.

Dynamic monitoring of intestinal microflora in the orally infected ducklings

Obvious changes of ERIC-PCR bands of orally inoculated ducklings were not observed until 24 h p.i. The numbers of the ERIC-PCR bands gradually decreased from 24 h to 72 h p.i. and reached the least at 72 h p.i. Thereafter, with the development of disease, the band numbers gradually increased until 6 d p.i. Two major bands (about 300 bp and 500 bp) were detected in each sample (Figure 1B).

Results of DNA fragments sequencing from major bands

The sequences about 500 bp of jejunum sampled at 72 h p.i., 100 bp of duodenum sampled at 6 d p.i. of healthy ducklings and 500 bp of ileum sampled at 72 h p.i., and 300 bp of duodenum sampled at 24 h p.i. of orally infected ducklings were analyzed with the BLAST program at the NCBI website (http://www.ncbi.nlm.nih.gov/blast) (Table 1).

Table 1 Sequencing results of DNA fragments from major bands

Major bands of health ducklings		Major bands of infected ducklings	
100 bp (6 d duodenum)	**500 bp (72 h jejunum)**	**300 bp (24 h duodenum)**	**500 bp (72 h ileum)**
Sinorhizobium meliloti enterobacterial, Pseudomonas syringae, *Escherichia coli* 536, UTI89, 301. (≥ 95%)	*Sinorhizobium meliloti* enterobacterial, *Xanthomonas campestris* enterobacterial, *Erwinia pyrifoliae* (≥ 90%)	*Yersinia enterocolitica subsp*, enterocolitica 8081 complete genome, *Escherichia coli* W3110 (APECO1, CFT073, L0001, UTI89, 536, K12-MG1655), Shigella dysenteriae Sd197, Shigella flexneri 8401 strain, Salmonella typhimurium LT2, Pseudomonas syringae pv. Phaseolicola, Pseudomonas syringae pv. glycinea, strain CYL325, Salmonella enterica subsp (≥ 90%)	*Sinorhizobium meliloti* enterobacterial, *Xanthomonas campestris* enterobacterial, *Yersinia pestis* (≥ 80%)

DISCUSSION

Methods to analyze intestinal microbial community

The structural dynamics of microbial communities have been monitored using amplified sequences or total genomic DNA and a variety of genetic fingerprinting techniques, such as Terminal Fragment Length Polymorphism (T-RFLP), Amplified Ribosome DNA Restriction Analysis (ARDRA), Denaturing Gradient Gel Electrophoresis/ Temperature Gradient Gel Electrophoresis (DGGE/ TGGE), of amplified partial 16S/18S rDNAs or fragments of conserved functional genes and Random Amplified Polymorphic DNA (RAPD), or Arbitrarily Primed PCR (AP-PCR)[18-21]. These techniques provide genomic patterns or profiles of microbial communities, in which the number of bands reflects the number of predominant community members, while the intensity of bands theoretically reflects population levels. ERIC sequence was first described in Escherichia coli, Salmonella typhimurium and other enterobacteria[22]. Two opposing primers were designed by Versalovic *et al*[16] in 1991 based on the 44-bp entire conserved central core inverted repeat (ERICALL). PCR amplification of bacterial genomic DNA with this primer pair results in highly reproducible and unique banding patterns for different genomes. ERIC-PCR has been widely used for typing of bacterial genomes based on the strain-specific fingerprints. In this study, ERIC-PCR was used to fingerprint the microbial community of fecal samples of research subjects to monitor intestinal microbial community diversity and dynamic change of healthy ducklings and infected ducklings with *S. enteritidis*. The results indicated that intestinal microbial community diversity and dynamic change are closely correlated with the process of infection. The numbers of ERIC-PCR bands of orally inoculated ducklings gradually decreased firstly, and then, with the development of disease, the band numbers gradually increased and recovered.

Distribution and stability of normal flora in intestinal tract

A large number of microbial species exist in gastrointestinal tract. Intestinal microflora plays an important role in digestive function, immunity and disease resistance. In this study, the diversity and dynamics of intestinal microbial community did not change obviously in control healthy ducklings analyzed with ERIC-PCR, which is consistent with the study that the quantity and proportion of each kind of bacteria are steady relatively[23]. The numbers of the bands formed by ERIC-PCR were the largest in healthy caecum that contained a bacterial content of 10^{10} cfu/mL-10^{12} cfu/mL[24]. The reason why the ERIC-PCR band numbers in rectum were the least and its microbial community were not steady is that the quantity and category of microflora were mainly influenced by the proportion of bacterium in ileum and cecum[25]. Duodenum and jejunum are transition zone of intestinal tract, and bacterium would be washed away to ileum[26]. So bands of duodenum and jejunum formed by ERIC-PCR were relatively less compared with that in jejunum and caecum.

Intestinal microbial community diversity and dynamic change of ducklings infected with oral S. enteritidis

The change of intestinal internal environment can result in dysbacteriosis. So-called dysbacteriosis, is that the quantity, category and proportion of each kind of bacteria changed, which will affect the function of host's gastrointestinal tract[27]. In this study, ERIC-PCR bands of orally inoculated ducklings did not change obviously until 24 h p.i. These results may suggest that the infection of *S. enteritidis* did not seriously affect intestinal microbial community of infected ducklings. However, the numbers of the ERIC-PCR bands gradually decreased from 24 h to 72 h p.i. This result may imply that intestinal microbial community diversity was affected by the continuous replication of *S. enteritidis* in the intestine of infected ducklings, which reflect the typical clinical sign of diarrhea and several pathological lesions of hyperaemia and haemorhage of the intestine. However, with the development of disease, the band numbers gradually increased until 6 d p.i., and the typical clinical signs and several pathological lesions also returned to almost normal at this time. Taken together, these data suggest that the stability of intestinal microbial community closely correlated with the progression of *S. enteritidis* infection.

Prominent bacterium in intestinal tract of S. enteritidis infected ducklings

Anaerobic bacterium is prominent in intestinal normal flora. In this study, the sequencing results of DNA fragments from major bands of the control healthy

ducklings indicated that all these bacteria were anaerobe or facultative anaerobe and among these bacteria, *Escherichia coli* and *Erwinia* play an important role in maintaining the balance of intestinal microecology. However, few data are available in the literature showing the other bacteria with ERIC sequence were prominent bacteria in intestinal tract of fowl; further investigations are needed to clarify this. Enteric infection result in the proportion of aerobe and facultative anaerobe increase[28-30]. In this study, sequence analysis showed that the nucleotide sequence homologies of the fragment about 500 bp of infected ileum sampled at 72 h p.i. were above 80% with S*inorhizobium meliloti enterobacterial*, *Xanthomonas campestris enterobacterial*, *Yersinia pestis*, and the nucleotide sequence homologies of the fragment about 300 bp of infected duodenum sampled at 24 h p.i. were more than 90% with *Yersinia enterocolitica subsp*, *enterocolitica* 8081, *Escherichia coli W3110* (*APEC O1, CFT073, L0001, UTI89, 536, K12-MG1655*), *Shigella dysenteriae Sd197*, *Shigella flexneri 8401 strain*, *Salmonella typhimurium LT2*, *Pseudomonas syringae pv. phaseolicola and Pseudomonas syringae pv.glycinea.strain CYL325*, *Salmonella enterica subsp*, *Erwinia amylovora*. However, among these bacteria, they are all pathogenic strains able to make host dysentery except *Escherichia coli K-12*, *MG1655*. These data suggest that the pathogenic bacteria were prominent in intestinal microbial community when orally infected ducklings with *S. enteritidis* developed signs of diarrhea. Moreover, several pathogenic bacterial species including *Yersinia pestis* represent a real secondary infection, which may imply that infected ducklings with *S. enteritidis* were followed by secondary bacterial infection.

The lesions of intestinal mucous membrane caused by *SE* infection result in dysbacteriosis of intestinal normal flora, the increase of native bacterium, and the transformation from beneficial bacterium to pathogenic bacterium. Furthermore, as a result of *SE* infection, depression of immunity, disfunction of intestinal mucous membrane and dysbacteriosis can also raise the pathogenic bacterial levels in the intestine, such as *Escherichia coli*, *Shigella* and *Salmonella*. Therefore, the increased proportion of pathogenic bacteria may be crucial in determination of diarrhea symptoms in *S. enteritidis* infection.

COMMENTS

Background

Salmonella has been a concern of public health for over 100 years and the incidence of Salmonella infections has increased dramatically, especially *S. enteritidis* infection. Knowledge about the Salmonella infection could be an additional means for decreasing the incidence of infection. Infection with Salmonella is usually started by oral ingestion of the pathogen and is followed by dysbacteriosis of the gut and diarrhea. It is therefore, necessary to understand intestinal microbial community diversity.

Research frontiers

Up to date, there has been no report about the intestinal microbial community diversity of animal *S. enteritidis* infected. ERIC-PCR, as a technique used for typing of bacterial genomes based on the strain-specific fingerprints obtained, will be an ideal method to study the intestinal microbial community diversity of *S. enteritidis* infected animals.

Innovations and breakthroughs

Previous studies considered that *S. enteritidis* established itself within the walls of the small intestine before a systemic infection could be demonstrated. However, these works were unable to identify the dynamic change of intestinal microbial community of *S. enteritidis* infected animals. In this study, the authors offered a significant improvement over current studies and suggested that *S. enteritidis* infection can cause dysbacteriosis of gastrointestinal tract.

Applications

This study will provide significant data for clarifing intestinal microbial community diversity of ducklings infected with *S. enteritidis* and new insight into the prevention and treatment of *S. enteritidis* infection.

Terminology

Dysbacteriosis**:** The change in quantity, category and proportion of each kind of bacteria.

Peer review

This study analyzed intestinal microbial community diversity of different segments of the gastrointestinal tract in healthy and orally infected ducklings with Salmonella enteritidis by ERIC-PCR method. The results show that SE infection can result in dysbacteriosis of infected ducklings, and the ERIC-PCR method is a useful tool when applied to pathogenesis and prevention of *S. enteritidis* infection.

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RAPID COMMUNICATION

KAI1 is a potential target for anti-metastasis in pancreatic cancer cells

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Abstract

AIM: To investigate whether KAI1, as a metastasis suppressor gene, is associated with invasive and metastatic ability of pancreatic cancer cells.

METHODS: KAI1 gene was transfected into pancreatic cancer cell line MiaPaCa Ⅱ by liposomes selected with G418. Expression of transfected cells was measured by Western blotting, immunofluorescence and immunocytochemistry. Tumor cell invasion and metastatic ability were detected through gelatinase activity and reconstituted basement membrane (Matrigel) assay. pCMV-KAI1 was directly injected into the heterotopic human pancreatic adenocarcinoma successfully established in the groin of BALB/C nude mice, by subcutaneous injection of MiaPaCa Ⅱ pancreatic cancer cells. The statistical analysis between groups was determined by Student's two tailed *t* test.

RESULTS: By Western blotting, MiaPaCa Ⅱ cells transfected by KAI1 gene indicated KAI1 expression at approximately 29.1 kDa. Cytoplasm staining was positive and uniformly spread in transfected cancer cells, using immunohistochemistry and immunofluorescence. The most obvious difference was present after 30 h (MiaPaca Ⅱ 43.6 ± 9.42, pCMV-MiaPaca Ⅱ 44.8 ± 8.56, pCMV-KAI1-MiaPaca Ⅱ 22.0 ± 4.69, $P < 0.05$). Gelatinolysis revealed a wider and clearer band of gelatinolytic activity in non-transfected than in transfected cells (MiaPaCa Ⅱ cells 30.8 ± 0.57, transfected cells 28.1 ± 0.65, $P < 0.05$). *In vivo* tumor growth rates of KAI1 transfectants with KAI1-Lipofectamine 1.22 ± 0.31 in A group were lower than control 4.61 ± 1.98 and pCMV-KAI 11.67 ± 0.81. Analyses of metastases with and without KAI1 transfection in mice were different in liver and lung between controls 1.62 ± 0.39, 0.45 ± 0.09, pCMV-KAI 1.01 ± 0.27, 0.33 ± 0.09 and KAI1-Lipofectamine 0.99 ± 0.21, 0.30 ± 0.09 respectively ($P < 0.05$).

CONCLUSION: High expression of KAI1 gene was found in transfected MiaPaCa Ⅱ human pancreatic cancer cells with lower metastatic ability. KAI1 gene plays an important role in inhibiting metastasis of pancreatic cancer after direct injection into pancreatic adenocarcinoma. These results show that the suppressed invasion and motor function of pancreatic cancer cells may be a key reason why the KAI1 gene controls pancreatic cancer cell metastasis.

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Key words: KAI1; Pancreatic cancer cell line; Transfection; Immunocytochemistry; Western blotting; Immunofluorescence; Gelatinolysis

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INTRODUCTION

Invasion and metastasis are remarkable characteristics in pancreatic cancer. During invasion and metastasis, primary pancreatic cancer cells have to move out, invade adjacent tissue, and circulate to distant organs where they may form new colonies[1]. Tumor invasion encompasses the process of tumor cell penetration or infiltration into adjacent tissue; therefore, metastasis depends on host immunological ability, local and systemic expansion of the tumor mass, and angiogenesis[2]. Once metastatic cells settle in a distant organ, nutrients and spaces are no longer

limited because newly formed metastases arise as islets of cancer cells surrounded by normal host cells. Invasion and metastasis are complex processes that involve molecular mechanisms, which lead to multiple gene alterations in pancreatic carcinoma, including alterations to KAI1, K-ras, DPC4 and P53[3-6]. KAI1 was originally identified as a metastasis suppressor gene.

KAI1 has been mapped to human chromosome 11p11.2. The expression level of KAI1 mRNA in human cell lines derived from metastatic prostate cancers is undetectable or much lower than that in normal prostate tissue[7]. Moreover, the gene transfer of KAI1 into rat AT6.1 prostate cancer cells results in significant suppression of pulmonary metastases. KAI1, which is identical to CD82, belongs to a structurally distinct family of membrane glycoproteins named transmembrane 4 (TM4). KAI1 is expressed ubiquitously, not only in prostate tissue, but also in a wide variety of other tissues, which suggests that it may play a role in suppressing metastases of other tissue-derived cancers, such as pancreatic, non-small cell lung, bladder, gastric and breast cancer[8-12]. These findings, taken together with the fact that KAI1 protein has a nearly ubiquitous tissue distribution, suggest that KAI1 may function as a metastasis suppressor gene in other types of cancers.

In our previous studies, we have demonstrated that KAI1 expression, at both mRNA and protein levels, is inversely correlated with the metastatic potential of some established human pancreatic cancer cell lines. In addition, we have also assessed KAI1 protein expression in human pancreatic cancer specimens from patients with known clinical outcome. We have found that more malignant tumor types express significantly lower levels of KAI1 protein, and down regulation of KAI1 at the RNA level is correlated with lymph node and distant metastases in patients with advanced pancreatic cancer[12,13]. The function of KAI1 with anti-metastasis has been unclear up till now. The aim of this study was to examine the expression of KAI1 protein in pancreatic cancer cells transfected by the gene, and to determine whether KAI1 transfected into pancreatic cancer cells can suppress invasive and metastatic ability of the cancer cells.

MATERIALS AND METHODS

Cells and cell culture

The human pancreatic cancer cell MiaPaCa Ⅱ was kindly provided by Dr. H. Friess. The cells was cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 IU/mL penicillin G and 100 μg/mL streptomycin, and passaged with 0.25% trypsin.

Transfection

Expression plasmids encoding KAI1 (pCMV-KAI1 DNA) were obtained from Dr. J. Dong (University of Virginia Health System). The day before transfection, cells were trypsinized and counted, and plated at 1×10^5 cells per well, so that they were 80%-90% confluent on the day of transfection. Cells were plated in 0.5 mL normal growth medium. For each well of cells to be transfected, we diluted 1.0 μg pCMV-KAI1 DNA into 50 μL medium without serum (this can be prepared in bulk for multiple wells). For each well of cells, we diluted 1 μL LF2000 Reagent (GibcoBRL, Maryland, USA) into 50 μL medium without serum and incubated for 5 min at room temperature. The diluted DNA was combined with the diluted LF2000 Reagent, and incubated at room temperature for 20 min. We added 0.5 mL medium without serum, then 100 μL DNA-LF2000 Reagent complexes was added directly to each well and mixed gently by rocking the plate back and forth. The cells were incubated at 37℃ in a CO_2 incubator for 24 h, and passaged at a 1:10 into fresh growth medium. After 24 h, we added 400 μg/mL G418 selection medium for the cells transfected by KAI1. At 4 wk after transfection, stable transfected cells were obtained. MiaPaCa Ⅱ cells transfected with pCMV served as a negative control.

Western blotting analysis

1×10^7 cells for Western blotting were prepared in 0.5 mL Lysis Buffer (10 mmol/L Tris/HCl, 5 mmol/L EDTA, 50 mmol/L NaCl, 30 mmol/L Na_3PO_4, 50 mmol/L NaF, 0.1 mmol/L Na_3VO_4, 1 mmol/L PMSF, 5 mg/mL aprotinin, 0.1% Triton X-100, pH 7.6). Homogenates were centrifuged for 10 min at 10000 rpm at 4℃. The supernatant was collected and maintained at -20℃. After denaturing at 100℃ for 5 min, the supernatant was subjected to SDS-PAGE on 5% concentrated gels and 7.5% segregated gels. Separated proteins were electrophoretically transferred to PVDF membranes for 1.5 h at 4℃. These membrane transfers were dried and incubated over night at 4℃ with 5% defatted milk powder/TBS/0.1% Tween-20. After washing with TBS, the membrane transfers were incubated with purified rabbit polyclonal antibody against KAI1 (CD82) (BD Biosciences, New Jersey, USA) 1:200 in buffer (10 mmol/L Tris/HCl, 100 mmol/L NaCl, 5% BSA, and 0.1% Tween-20, pH 7.4). Following three washes of 20 min in TBS/Tween-20, membrane transfers were incubated with goat anti-rabbit IgG/horseradish peroxidase conjugate for 1 h. Each was removed by three 20 min washes in TBS/Tween-20. Immunoreactive proteins in peroxidase-treated transfers were visualized using an ECL Western blotting detection system (Amersham Life Sciences, Little Chalfont, United Kingdom) with exposure to X-ray film for 30-120 s. Prestained SDS-PAGE standard proteins (Bio-Rad, Richmond, CA, USA) were employed as molecular size markers.

Immunofluorescence

The pancreatic cells before and after transfection were allowed to attach overnight to chambered glass slides. After incubation, cells were rinsed in PBS, fixed in ice-cold methanol, washed, and blocked in a PBS solution containing globulin-free BSA (1%), Triton X-100 (0.3%) and goat serum (3%). The cells were incubated with primary antibodies (CD82, 1:80 dilution) in a humidified

chamber for 1 h at room temperature, washed in PBS, and incubated again for 1 h in the dark with a secondary antibody (1:80 dilution) coupled to the fluorophore. After washing, the cell lines were covered with DAPI solution (Roche, New Jersey USA). Slides were mounted with coverslips and examined by fluorescence microscopy.

Immunocytochemical detection of KAI1 protein

Briefly, cells were washed in PBS, fixed in ice-cold acetone for 20 min at 4℃, soaked in 0.3% H_2O_2 for 30 min, and blocked in a PBS solution containing NP40 and goat serum (10%). The cells were incubated with specific KAI1 antibody, diluted 1:20 in PBS containing 10% goat serum and NP40, at 4℃ overnight. After washing, we added a secondary antibody (1:100 dilution), coupled to biotin for 1 h at room temperature, and the samples were stained with DAB.

Cell invasion assay

In Transwell cell culture chambers (Costar 3422, Cambridge, MA, USA), polycarbonate membranes with 8.0-μm pore size were spread with 40 μL (10 μg) of Matrigel (BD Biosciences) on the upper surface at 37℃. After 1 h, each insert was placed in 500 μL DMEM containing 0.1% BSA, for hydration at 37℃ for 30 min. The membranes were precoated with 40 μL (2 μg) fibronectin (Invitrogen) on the lower surface of the filters. The filters were dried at room temperature for 45 min. MiaPaCa Ⅱ and KAI1-MiaPaCa Ⅱ cells (1×10^5) suspended in 100 μL DMEM containing 0.1% BSA were added to the upper compartment and incubated at 37℃ for 10-60 h. Every 10 h, the filters were fixed with 95% ethanol for 15 min and stained with hematoxylin and eosin. After gently rinsing with water, the cells on the upper surface of the filters were removed by wiping with a cotton swab. The filter containing the stained cells that invaded to the lower surface was manually counted under a microscope at × 400 magnification. Each assay was performed in triplicate.

Gelatin zymography

MiaPaCa Ⅱ and KAI1-MiaPaCa Ⅱ cells (1×10^5) were cultured overnight using ordinary serum-containing DMEM, washed with serum-free DMEM, and further cultured using serum-free DMEM for 24 h. The supernatant was collected, and the cellular fragment was removed by low-speed centrifugation and concentrated 30-fold with a membrane dialysis concentrator. The conditioned medium was used for the detection of gelatinase activity. Gelatin zymograms were obtained according to the method reported by Heussen and Dowdle[18], with some modifications. In brief, the conditioned medium was resolved by SDS-PAGE using 8% separating gel containing 0.1% (w/v) gelatin and 5% stacking gel. The gels were washed twice in 2.5% (w/v) Triton X-100 for 30 min at room temperature to remove SDS, then incubated in reaction buffer containing 50 mmol/L Tris/HCl, pH 7.6, 10 mmol/L $CaCl_2$ and 1 μmol/L $ZnCl_2$ for 12-16 h at 37℃. The gelatin gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid for 4 h. After destaining with 45% methanol and 10% acetic acid, the gelatinolytic activity on the gel was detected as clear bands on a blue background of undigested gelatin, and quantitated using a Master Scan gel analysis system (Scanalytics, Billerica, MA, USA).

In vivo growth and experimental metastasis assay

To investigate the effect of KAI1 on *in vivo* metastasis, 1×10^6 cells in 0.1 mL 0.9% NaCl were injected subcutaneously into the flank region of 36 nu/nu mice (20 g body weight; China Medical University Breeding Laboratories, Shenyang, China). Mice were randomly divided into two groups: group A ($n = 18$) was injected with the KAI1 gene 10 d after injection of pancreatic cancer cells, and group B ($n = 18$) was treated 21 d after injection as our report[14]. The mice in group A were randomly divided into three (six mice per group), including pCMV-KAI1, pCMV-KAI1-Lipofectamine and control. Group B was divided the same as group A. One hundred micrograms of recombinant plasmid pCMV-KAI1, 100 μg pCMV-KAI1-Lipofectamine (1:1) and 100 μL 0.9% NaCl were directly injected into pancreatic adenocarcinoma every 7 d, and repeated three times. Tumor size was monitored 1 wk after inoculation of tumor cells. Tumor volume was determined using the formula $V = A \times B \times 0.4$, where A and B are the larger and smaller diameters of the tumor, respectively. After 50 d, the mice were necropsied, and the lungs were removed. Visible lung metastases were counted in Bouin's fixed tissues, with the aid of a dissecting microscope.

Statistical analysis

The statistical significance of differences between the groups was determined by applying Student's two-tailed *t* test. $P < 0.05$ was considered statistically significant. Values are indicated as the mean ± SD.

RESULTS

Confirmation of KAI1 gene transfection

The pCMV-KAI1 DNA was transformed into CM109 using the Maxi preparation. KAI1 DNA was identified in the correct position. The correct orientation was confirmed by PCR and sequencing analysis. MiaPaCa Ⅱ cells transfected with pCMV-KAI1 DNA retained G418 resistance for 10 generations. The transfection of MiaPaCa Ⅱ cells indicated KAI1 expression.

Expression of KAI1 protein

The stably transfected cells were analyzed by Western blotting to evaluate the intracellular amounts of KAI1 protein. The major specific KAI1 protein was clearly visible at about 29.1 kDa in MiaPaCa Ⅱ pancreatic cells transfected with pCMV-KAI1 DNA, but almost undetectable in cells transfected with pCMV. The most positively staining was found in the cytoplasm in transfected cancer cells. Negative

Table 1 Invasive ability of pancreatic cancer cells (mean ± SD)

Cell type	Number of cells at different times					
	10 h	20 h	30 h	40 h	50 h	60 h
MiaPaca Ⅱ	20.6 ± 6.43	23.6 ± 7.50	43.6 ± 9.42	59.8 ± 6.06	86.8 ± 4.32	117.4 ± 4.88
pCMV-MiaPaca Ⅱ	21.0 ± 7.21	24.8 ± 6.46	44.8 ± 8.56	62.6 ± 3.11	87.4 ± 1.82	115.4 ± 3.58
pCMV-KAI1-MiaPaca Ⅱ	14.8 ± 6.69	16.4 ± 4.51	22.0 ± 4.69	25.6 ± 5.55	29.4 ± 4.93	33.6 ± 6.11
	$P > 0.05$	$P > 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

$P < 0.05$ between KAI1-transfected and non-transfected cells.

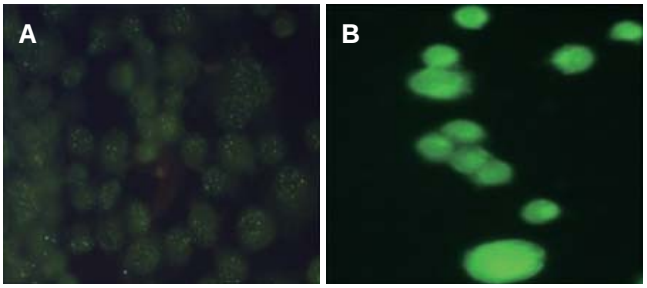


Figure 1 **A**: Very weak immunofluorescence in cells with empty plasmids without KAI1; **B**: Strong green immunofluorescence in cells transfected with KAI1.

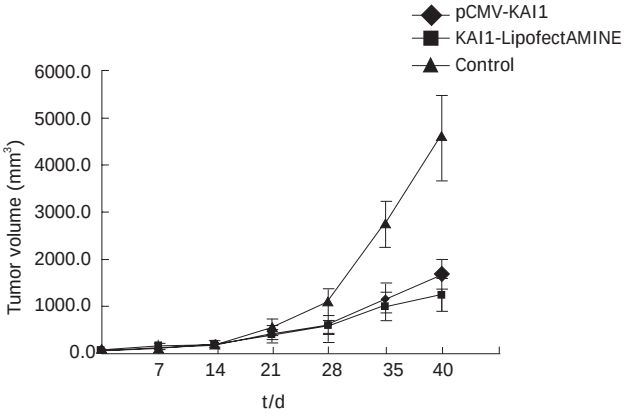


Figure 2 Effect of KAI1 expressions on *in vivo* tumor growth in group A.

staining intensity in non-transfected cells was shown to be comparable with that in transfected cells. Strong green immunofluorescence (Figure 1) was seen in the transfected MiaPaCa Ⅱ pancreatic cancer cells, whereas very weak immunofluorescence was found in cells transfected with pCMV.

Invading ability of cells transfected with KAI1

The transfection of KAI1 cells on tumor invasion of Matrigel was evaluated using a Transwell chamber assay. The data in Table 1 shows that the stained cells invading the lower surface of the filters of pCMV-KAI1-MiaPaca Ⅱ were less than pCMV-MiaPaca Ⅱ and MiaPaca Ⅱ cells without transfection. There was no distinct difference in the number of invasive cells before and 10 and 20 h after transfection, while there was an obvious difference after 30 h ($P < 0.05$). There was no difference in the number of invasive cells observed between cells transfected by pCMV vector control and MiaPaca Ⅱ ($P > 0.05$). The results show that transfection of the KAI1 gene significantly reduced the invasive ability of MiaPaCa Ⅱ cells.

Table 2 Effect of KAI1 expression on *in vivo* tumor growth (mean ± SD)

	Group	*n*	Tumor volume (cm^3)	*t*	*P*
	Control	6	4.61 ± 1.98	-	-
A	KAI1-LipofectAMINE	6	1.22 ± 0.31	2.860	< 0.05
	pCMV-KAI1	6	1.67 ± 0.81	2.414	< 0.05
	Control	6	4.61 ± 1.98	-	-
B	KAI1-LipofectAMINE	6	4.87 ± 1.58	0.199	> 0.05
	pCMV-KAI1	6	4.16 ± 2.81	0.286	> 0.05

$P < 0.05$ between KAI1 transfection and controls.

Gelatinolysis in MiaPaCa Ⅱ pancreatic cells

We investigated the effect of transfection on the gelatinolytic activity of MMP-2 by gelatin zymography. Zymograms from MiaPaCa Ⅱ cells revealed a wider and clearer band of gelatinolytic activity than that from transfected cells. Values of I A were 30.8 ± 0.57 of MiaPaCa Ⅱ cells, moreover 28.1 ± 0.65 of transfected cells ($P < 0.05$).

Tumor growth

Tumor volume was assessed at 7 d after injection of tumor cells and continuously every 3 d until 50 d. Time curves showing the percentage increase in tumor size in saline-treated and KAI1-treated mice are shown in Figure 2. At d 21, the KAI1 transfectants had significantly lower tumor volume compared with the controls ($P < 0.05$). As a result of KAI1 administration, tumor growth was dramatically reduced in pCMV KAI1 in group A.

In group A (Table 2), the tumor volume inhibitory rate of the KAI1 and KAI1-Lipofectamine groups was 63.79% and 73.51%, and the lung metastatic nodule number was 0.5 ± 0.83 and 2.33 ± 1.63, respectively. The tumor volume, lung weight, liver weight and lung metastatic nodule number of the KAI1-treated group were significantly lower than those in the control group ($P < 0.05$; Table 3). These data showed no significant difference between the KAI1 and KAI1-Lipofectamine groups. In group B, there was no significant difference between the gene therapy and saline control groups. There was a significant difference in tumor volume, lung weight, liver weight and lung metastatic nodule number between group A and B ($P < 0.05$).

Table 3 Analyses of liver and lung metastases with and without KAI1 transfaction in mice *in vivo* (mean ± SD)

	Group	*n*	Liver weight (g)	*t*	*P*	Lung weight (g)	*t*	*P*
	Control	6	1.62 ± 0.39			0.45 ± 0.09		
A	KAI1-LipofectAMINE	6	0.99 ± 0.21	2.661	< 0.05	0.30 ± 0.09	3.181	< 0.05
	pCMV-KAI1	6	1.01 ± 0.27	2.295	< 0.05	0.33 ± 0.09	2.291	< 0.05
	Control	6	1.62 ± 0.39			0.45 ± 0.09		
B	KAI1-LipofectAMINE	6	1.69 ± 0.17	0.312	> 0.05	0.38 ± 0.03	1.348	> 0.05
	pCMV-KAI1	6	1.46 ± 0.28	1.286	> 0.05	0.36 ± 0.04	1.956	> 0.05

$P < 0.05$ between KAI1 transfection and controls.

DISCUSSION

The KAI1 gene as a metastasis suppressor gene may be a useful marker for the metastatic/invasive potential of several human tumor types[9-11,15]. In the case of pancreatic cancer, KAI1 appears to be crucial for the development of distant organ metastasis, since loss of KAI1 expression is found in lymph node and distant metastases[12,13,16], therefore, down-regulation of the KAI1 gene during cancer development may be associated with methylation of the promoter or *p53* regulation, but does not commonly interfere either mutation or allelic loss of the KAI1 gene[17]. Although the mechanisms of invasion and metastasis of the KAI1 gene have not been elucidated in pancreatic and other cancers, a better understanding of these mechanisms that lead to cancer remains a major goal because it may help develop strategies for earlier diagnosis and better treatment.

In the present study, KAI1 expression was examined using immunohistochemistry. Positive staining for the protein in cells transfected with the KAI1 gene should be observed mainly at the cell membrane, because KAI1 is a member of the TM4 superfamily, however protein localization in the transfected cells was mainly found in the cytoplasm, similar to that in previous studies of human pancreatic cancer[18]. We used a rabbit polyclonal antibody and found that KAI1 was expressed abundantly in transfected cells, but there was negative staining in the non-transfected cells. Our data suggests that the protein upregulation in KAI1 transfected cells is the same as the expression in human pancreatic cancer samples. This may suggest that KAI1 inhibits metastasis in pancreatic cancer cells associated with the human samples[12].

Cancer mortality is due largely to distant metastases and subsequent organ failure. Metastasis involves the degradation of the basement membrane and stromal extracellular matrix (ECM) and migration into adjoining blood vessels, which results in tumor growth in distant organs[19,20]. Degradation of the basement membrane and ECM involve the secretion of several proteases, such as one or more members of the MMP family[21-23]. Among more than 20 MMPs that have been identified[24], MMP-2 has been described as a negative prognostic marker of metastasis and disease-free interval[25-27]. We transfected KAI1 cDNA into highly metastatic pancreatic cancer cells, MiaPaCa Ⅱ, and investigated whether KAI1 gene reduced invasive ability of transfected cells and decreased gelatinase activity. In other words, the gelatinolytic activity by gelatin zymography was shown to increase in cells that were not transfected with the KAI1 gene. Our results showed that after KAI1 gene transfection, the activity of gelatinase decreased markedly, which suggests that KAI1 may suppress expression of gelatinase. Through a reconstituted basement membrane (Matrigel) *in vitro*, we found that stained cells invading the lower surface of the filters of pCMV-KAI1-MiaPaca Ⅱ were less than pCMV-MiaPaca Ⅱ and MiaPaca Ⅱ cells without transfection. The number of invasive cells observed did not obviously differ between cells transfected with the pCMV vector and non-transfected cells. This indicates that, as with any malignancy, the propensity for local/regional invasion and distant metastasis of pancreatic cancer may be based on its ability to invade the basement membrane.

The time of gene therapy selected was at 10 d (group A) and 21 d (group B) after injecting pancreatic cells, according to our previous research[14]. In agreement with studies of prostate cancer, colon cancer and melanoma cells[7,28,29], our data also indicated that KAI1 expression suppressed metastasis *in vivo* when transfected into pancreatic cancer cells, and revealed a decrease in tumor volume of KAI1 transfectants 3 wk after inoculation, similar to breast cancer cells[11]. These results showed that KAI1 cDNA may be directly injected into the body of pancreatic adenocarcinoma to inhibit its metastasis and growth, without being mediating by liposomes, but the inhibition distinctly correlated with the time at which KAI1 gene was injected. The present data, along with our previous results[30], showed that the KAI1 gene may have play a key role in inhibiting metastasis of pancreatic cancer, by controlling cancer cell growth at secondary metastatic loci.

KAI1, as a metastasis suppressor gene, has drawn special attention as a new therapeutic target, in the hope that re-activation of the gene reduces the metastatic ability of malignant cells. We found that down-regulation of KAI1 mRNA level was correlated with lymph node and distant metastases in patients with advanced pancreatic cancer[11], whereas the results suggested that the activity of gelatinase, ability to invade the basement membrane, and metastasis of pancreatic cancer in mice, were decreased in the cells transfected with the KAI1 gene. In general, the results demonstrated that a decreased level of KAI1 mRNA expression in advanced pancreatic cancer involves both invasion and metastasis based on principles of cellular and molecular pathology.

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COMMENTS

Background

Metastasis is an important feature in pancreatic cancer. However, recently no new breakthroughs have been found. Therefore it is necessary to use new techniques to detect early-stage metastasis in pancreatic cancer.

Research frontiers

This is a frontier of the molecular biology of tumor genetics interfering with up-regulation of the KAI1 gene to inhibit growth and metastasis of primary pancreatic cancer in mice. The propensity for local and regional invasion and distant metastasis of pancreatic cancer may be associated with its ability to invade the basement membrane.

Innovations and breakthroughs

A leading phenomenon on controlling metastasis of pancreatic cancer was found in KAI1 gene regulation. It may suggest mechanisms for inhibiting metastases of other tumors in the human body.

Applications

The findings help to explain some of the mechanisms behind metastasis of cancer. This research may contribute to providing new gene treatment options for pancreatic cancer patients.

Peer review

This is a good study. The authors have shown that there was high expression of the KAI1 gene in transfected MiaPaCa II human pancreatic cancer cells with lower metastatic ability. They conclude that the KAI1 gene plays an important role in inhibiting the metastasis of pancreatic cancer.

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RAPID COMMUNICATION

Laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct and traditional open operation

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Abstract

AIM: To investigate the possibilities and advantages of laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct compaired with traditional open operation.

METHODS: Laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct and traditional open operation were performed in two groups of patients who had gallstones in the left lobe of liver and in the common bile duct. The hospitalization time, hospitalization costs, operation time, operative complications and post-operative liver functions of the two groups of patients were studied.

RESULTS: The operation time and post-operative liver functions of the two groups of patients had no significant differences, while the hospitalization time, hospitalization costs and operative complications of the laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration in the common bile duct group were significantly lower than those in the traditional open operation group.

CONCLUSION: For patients with gallstones in the left lobe of liver and in the common bile duct, laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct can significantly shorten the hospitalization time, reduce the hospitalization costs and the post-operative complications, without prolonging the operation time and bringing about more liver function damages compared with traditional open operation. This kind of operation has more advantages than traditional open operation.

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Key words: Laparoscopy; Fiber choledochoscopy; Hepatic lobectomy; Exploration of common bile duct

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INTRODUCTION

Since the first laparoscopic hepatic lobectomy performed by Reich in 1991, the laparoscopic operation procedure has been practiced in hepatobiliary surgery[1,2]. Because of the abundant blood supply for the liver and pneumoperitoneum, hemorrhage and gas embolism often occur during the operation[3]. Laparoscopic hepatic lobectomy is an operation procedure with high difficulties and risks[4]. However, in recent years, with the development of laparoscopic instruments and operative skills, laparoscopic hepatic lobectomy has gradually become a common operation procedure in clinical practice[5,6]. It has many advantages over the traditional open operation[7]. In traditional open operation, T-pieces should be placed in the common bile duct to drain the bile after exploration of the common bile duct, which provides channels for surgeons to take out the possible remnant gallstones. However, it also brings about heavy burdens on the patients, such as prolonged hospitalization time, unbalance between electrolytes and digestive dysfunction caused by bile drainage, local infection on the abdominal wall caused by the T-piece, complicated care for the T-piece. Researchers have tired to solve these problems for a long

time. In this study, we performed laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct in 10 patients with gallstones in the left lobe of liver and in the common bile from September 2005 to December 2005. At the same time, traditional open hepatic lobecomy and T-piece drainage were performed in 12 patients. The former operation procedure has significant advantages over the latter. The results are reported here.

MATERIALS AND METHODS

Clinical data

Twenty-two patients were definitely diagnosed having gallstones in the left lobe of liver and in the common bile duct by CT and operative exploration from September 2005 to December 2005. Among these patients, 9 were male and 13 were female. Their age ranged from 29 to 58 (average 44) years and their liver functions were normal (Child grade A). Eleven patients had no icterus. The total bilirubin level ranged from 20 to 100 μmol/L in 8 patients, and was higher than 100 μmol/L in 3 patients.

Operation methods

In ten patients, ligments of the liver were dissociated by laparoscopic instruments under pneumoperitoneum condition. The trocar puncture point near the xiphoid was extended to about 5 cm, from which the left lateral lobe of liver was resected and the calculi in the intrahepatic duct were taken out without pneumoperitoneum, the left intrahepatic duct and the common bile duct were examined under a fiber choledochoscope for the possible remnant gallstones. After the remnant gallstones were taken out and the duodenal papillae were validated to be normal. The open part of the left intrahepatic duct and the left liver section were sutured. Traditional open operation was performed in 12 patients. After the left lateral lobe of liver was resected and the remnant gallstones were taken out from the common bile duct, T-pieces were conventionally used to drain the bile.

Statistical analysis

Data were processed with the Systat software (SPSS11.0) for statistical computation and graphing. One-way analysis of variance (ANOVA), independent-sample *t* test and chi square test were used to evaluate the differences between groups. $P < 0.05$ was considered statistically significant.

RESULTS

Operation time and hospitalization time

There were no significant differences in the operation time of laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct and traditional open operation between the two groups. There were no significant differences in the time of hepatic inflow occlusion during the left lateral lobectomy between the two groups. While the hospitalization time and hospitalization costs of the laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration in the common bile duct group were significantly lower than those in the traditional open operation group ($P < 0.05$, Table 1).

Table 1 Operation time, hospitalization time and costs of different groups of patients (mean ± SD)

Comparative aspects	Laparoscopy and choledochoscopy group	Traditional open operation group
Operation time (h)	2.5 ± 0.3	2.0 ± 0.5
Time of hepatic inflow occlusion (min)	15.0 ± 3.0	10 ± 5.0
Hospitalization time (d)	5.0 ± 1.0	13.0 ± 3.0
Hospitalization cost (ten thousand yuan)	1.2 ± 0.3	1.5 ± 0.5

Table 2 Postoperative complications in 22 patients

Postoperative complications	Laparoscopy and choledochoscopy group	Traditional open operation group
Hemorrhage of hepatic incisal margin	0	0
Biliary fistula	0	1
Biliary tract stenosis	0	0
Biliary tract bleeding	0	0
Infection or exudation of abdominal fistulas	0	12

Postoperative complications

The incision in the laparoscopic and choledochoscopic group was much shorter than that in the traditional open operation group. During the operation, the common bile duct needed not to be cut open and T-piece needed not to be used to drain the bile. Postoperative complications such as biliary fistula, biliary tract bleeding and stenosis in the laparoscopic and choledochoscopic group were not as common as in the traditional open operation. Mild biliary fistula only occurred in one patient of the open operation group. A all T-pieces were safely pulled out from all patients of the open operation group one month after operation and the abdominal fistulae healed quite well (Table 2).

Postoperative liver function

There were no significant differences in the postoperative liver function between the two groups three and seven days after operation. However, the liver function in the two groups was better seven days than three days after operation ($P < 0.05$, Table 3).

DISCUSSION

Micro-wound operation is becoming the trend of surgery in the 21st century. Laparoscopic surgery is regarded as an important component part of micro-wound surgery, because of its advantages such as alleviate wound and rapid concrescence. Laparoscopy has been tried in many aspects of abdominal operation[8,9]. Both hepatic artery and portal vein give the blood supply for liver, hepatic inflow

Table 3 Postoperative liver functions in 22 patients (mean ± SD)

Postoperative liver functions	Laparoscopy and choledochoscopy group		Traditional open operation group	
	3 d after operation	7 d after operation	3 d after operation	7 d after operation
Albumin (g/L)	30.2 ± 5.0	40.0 ± 3.5	28.8 ± 4.2	41.0 ± 5.0
Total protein (g/L)	60.0 ± 4.0	75.0 ± 3.0	58.5 ± 5.0	70.5 ± 4.0
AKP (U/L)	90.5 ± 10.0	55.5 ± 7.0	80.0 ± 8.0	50.5 ± 5.0
ALT (U/L)	150.0 ± 32.0	50.0 ± 10.0	180.0 ± 42.0	55.0 ± 8.0
AST (U/L)	70.0 ± 5.0	20.3 ± 7.5	75.8 ± 8.0	18.5 ± 8.5
TBIL (μmol/L)	8.0 ± 2.5	5.0 ± 3.2	7.8 ± 3.5	5.2 ± 2.6
SBIL (μmol/L)	16.5 ± 3.0	8.4 ± 1.7	17.5 ± 2.5	9.1 ± 2.0

occlusion and suturing the hepatic incisal margin are usually used to control the bleeding during the open operation[10,11]. However, during laparoscopic hepatoectomy, it would be impossible to use these methods, since they can lead to bleeding more easily[12]. The negative pressure of the hepatic vein and the positive pressure of the abdominal cavity cause CO_2 gas embolism in the blood more easily during laparoscopic hepatoectomy[13,14]. Thus, laparoscopic hepatoectomy is considered a difficult operation with a high risk requiring high techniques[15,16]. In recent years, with the development of laparoscopic appliance and operative skills, laparoscopic operation is wildly performed in hepatobiliary surgery[17,18]. In traditional open common bile duct exploration, T-pieces are used for bile drainage and treatment of possible remnant gallstones[19]. However, it also brings about heavy mental burdens to the patients, such as prolonged hospitalization time, electrolytical and digestive unbalance caused by bile drainage and local infection of the abdiminal wall near the T-piece[20-22]. In our study, laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct was performed in 10 patients with gallstones in the left lobe of liver and in the common bile duct. Ligments of the liver were dissociated with laparoscopic instruments under pneumoperitoneum condition, which avoided the long incision of the ligments, and dissociation in traditional open operation. The trocar puncture point near the xiphoid was extended to about 5 cm, from which we resected the left lateral lobe of liver and took out the calculi in the intrahepatic duct without pneumoperitoneum, thus making it possible to use hepatic inflow occlusion for bleeding control and avoiding CO_2 gas embolism under pneumoperitoneum[23,24]. From the extended trocar incision and the left intrahepatic duct, the common bile duct was examined under fiber choledochoscope for the possible remnant gallstones. After the remnant gallstones were taken out and the duodenal papillae were found to be normal. We sutured the open part of the left intrahepatic duct and the left liver section, thus making the bleeding and biliary fistulae more easily to be controlled. In the laparoscopic operation[25], the wall of the common bile duct needs not to cut open, thus avoiding many common complications of open operation[26], such as biliary fistula, cholangitic stenosis, biliary tract bleeding, electrolytical or digestive unbalance and local infection[27,28]. In our study, the liver function of the two groups was changed. The liver function impairment of the laparoscopic group was milder than that of the traditional open operation group. These finding reflect the advantages of laparoscopic operation. As an importantt component of micro-wound surgery, laparoscopic operation is wildly used in many fields of surgery[29,30]. Because of its micro-wound and safety, it has gradually become the first choice of method for many operations[30]. In laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct, the much shorter insicion lessens the post-operative pain of patients and no T-piece drainage also markedly reduces the post-operative complications.

In conclusion, laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct has more advantages than traditional open operation.

COMMENTS

Background

Since the first laparoscopic hepatic lobectomy performed by Reich in 1991, the laparoscopic operation procedure has been practiced in hepatobiliary surgery. Because of the abundant blood supply for the liver and pneumoperitoneum, hemorrhage and gas embolism often occur during the operation, laparoscopic hepatic lobectomy is considered an operation procedure with high difficulties and risks. However, in recent years, with the development of laparoscopic instruments and operative skills, laparoscopic hepatic lobectomy has gradually become a common operation procedure in clinical practice and has many advantages over the traditional open operation.

Research frontiers

Micro-wound operation is becoming the trend of surgery in the 21st century and laparoscopic surgery is considered an important component part of micro-wound surgery. In this study, we investigated the possibilities and advantages of laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct over the traditional open operation.

Innovations and breakthroughs

In this study, laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct was performed in patients with gallstones in the left lobe of liver and in the common bile duct. Ligments of liver were dissociated with laparoscopic instruments under pneumoperitoneum condition. The trocar puncture point near the xiphoid was extended to about 5 cm, from which we resected the left lateral lobe of liver and took out the calculi in the intrahepatic duct without pneumoperitoneum. From the extended trocar incision and the left intrahepatic duct, the common bile duct was examined by fiber choledochoscopy for the possible remnant gallstones. After the remnant gallstones were taken out and the duodenal papillae were validated to be normal, we sutured the open part of the left intrahepatic duct and the left liver section.

Applications

In the laparoscopic hepatic left lateral lobectomy combined with fiber choledochoscopic exploration of the common bile duct, the much shorter insicion lessened the post-operative pain of patients and no T-piece drainage also markedly reduced the post-operative complications.

Peer review

This clinical study by Zhang *et al* describes a comparison between laparoscopic hepatic left lateral lobectomy *etc vs* traditional open operations. This manuscript is very informative and has impact the the methodology used for common bile duct exploration.

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CASE REPORT

Clear cell adenocarcinoma of the colon: A case report and review of literature

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Abstract

A primary clear cell adenocarcinoma of the colon is a rare oncologic entity. We herein report a case of such a tumor of the sigmoid colon in a 71-year-old woman who was successfully treated by an endoscopic polypectomy in our hospital. We also reviewed the published reports regarding cases of primary clear cell tumors in the colon.

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Key words: Adenoma-carcinoma sequence; Clear cell carcinoma; Colonic adenoma; Colon cancer; Endoscopic polypectomy

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INTRODUCTION

The first report of primary clear cell adenocarcinoma of the colon was published in 1964[1]. Even though advances have been made in the techniques regarding pathological examinations and immunohistochemistry over the past years, this tumor has been rarely diagnosed in the colon. We report a rare case of a clear cell adenocarcinoma in the colon which was successfully treated by an endoscopic polypectomy.

CASE REPORT

A 71-year-old Japanese woman presented at our hospital due to symptoms of lower abdominal pain and melena. After further examination, the pain was suspected to be due to ischemic colitis. After the symptoms of the ischemic colitis improved, we performed lower endoscopy to the cecum, which revealed healing ischemic lesions at the descending colon and a 7-mm diameter "berry-like" polyp in the sigmoid colon (Figure 1). The biopsy specimens of the polyp did not disclose a definite adenocarcinoma, but an adenoma instead. Radiologic examinations showed no evidence of a tumor elsewhere in the body, including the kidneys or the gynecologic regions. However, the surface of the polyp had a granular surface pattern and an irregular microcapillary appearance, which seemed to indicate a focal malignancy. Because endoscopic polypectomy alone for early polypoid colonic cancer is highly effective[2], we therefore performed a polypectomy during endoscopy in suspicion of focal malignancy. The resected "berrylike" polyp measured 8 mm × 6 mm in diameter with a granular lesion at the top with a cream colored portion in part.

The microscopic examination showed clear cell adenocarcinoma on the top of the polyp and adenoma at the base and body of the polyp. There was no transition between the areas of the usual adenoma and the clear cell adenocarcinoma. Vascular invasion and lymphatic involvement were not found. The cut margin was negative (Figure 2). As shown in Figure 2, we considered the polypectomy was a curative treatment. In PAS staining, the adenoma portion revealed PAS-positive mucin on the superficial layer, while the clear cell adenocarcinoma portion showed mildly PAS-positive mucin. In the Alcian blue staining, the adenoma took up the blue stain, while the clear cell adenocarcinoma did not (Figure 3). For immunochemical staining, the clear cell adenocarcinoma showed positive staining for p53, negative for CK7, weakly positive for CD10, and positive for CK20 (Figure 4), while the adenoma portion showed slightly less positive staining for p53 than did the clear cell carcinoma, and it was negative for CK7, weakly and focally positive for CD10, and positive for CK20. About 70% of the clear cell adenocarcinoma showed Ki67 positive staining, while the adenoma showed about 45% positive staining.

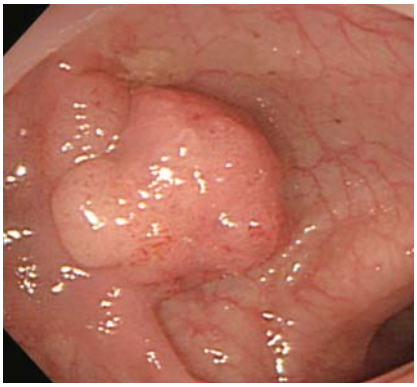


Figure 1 The 7-mm diameter "berrylike" polyp on the sigmoid colon.

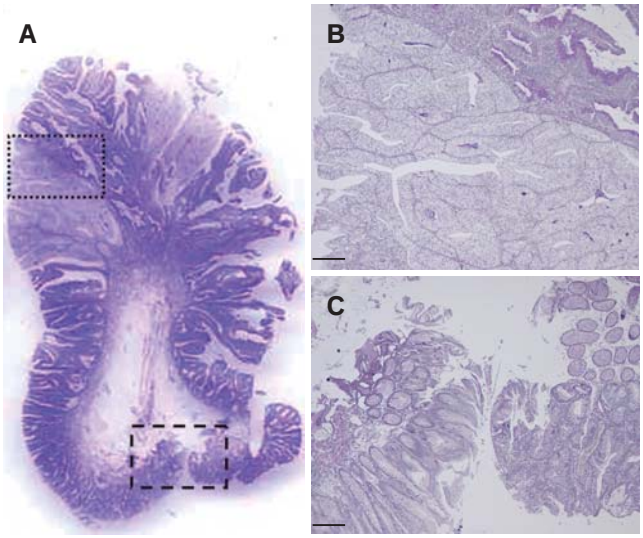


Figure 2 **A**: Polypectomy specimen containing adenoma and partial clear cell carcinoma. The cut margin was showed on the bottom side; **B**: There was no transition between the areas of the usual adenoma on right top of the figure and the clear cell adenocarcinoma on left bottom of the figure; **C**: The cut margin was showed normal colonic mucosa, indicating that this polypectomy was curative treatment. Each bar indicates 200 μm.

DISCUSSION

The presence of clear cell change in colorectal adenoma and adenocarcinoma is extremely unusual. The incidence of a clear cell change in the colorectal adenomas has been reported to be 0.086% out of 3486 cases[3]. In 1964, Hellstrom and Fischer described the first case of adenocarcinoma of the sigmoid colon, composed of neoplastic clear cells which resembled the physaliferous (clear) cells of chordomal tumors[1]. A clear cell adenocarcinoma generally develops in the kidneys[4-6], ovaries[7], extraovarian endometriosis[8,9], uterine cervix[10,11], upper vagina, or lower genital tract[12]. These organs originate from the mullerian system, which may explain the occurrence of clear cell adenocarcinoma in these systems[13]. Therefore, an isolated clear cell adenocarcinoma of the colon is a rare malignancy and its ontogeny remains unclear.

Regarding the clinical characteristics, more male patients with a clear cell adenocarcinoma of the colon have been reported, and the tumor tends to be located on the left side. Almost all cases in Table 1 were reported as an adenoma situated next to a clear cell adenocarcinoma,

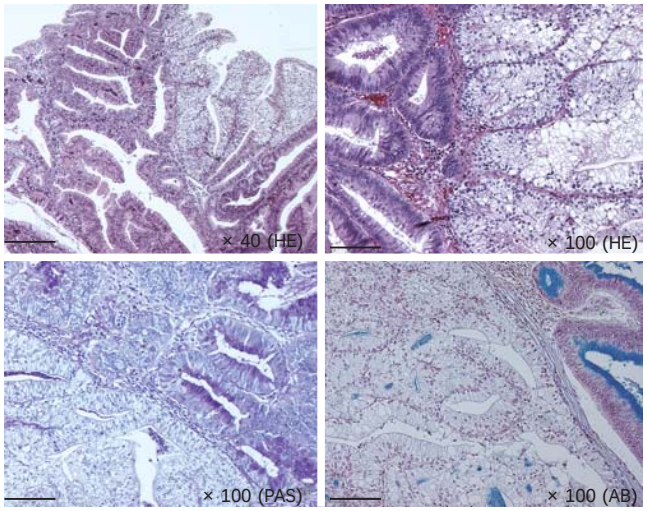


Figure 3 HE staining shows clear cell carcinoma with clear cytoplasm in a normal colonic adenoma (Two top figures). PAS staining shows purple staining on the adenoma on right of the figure, and mild purple staining on the clear cell carcinoma on left of the figure. Alcian blue staining shows granular mucin on the adenoma on right of the figure, and there are no staining on the clear cell carcinoma on left of the figure. Each bar indicates 200 μm.

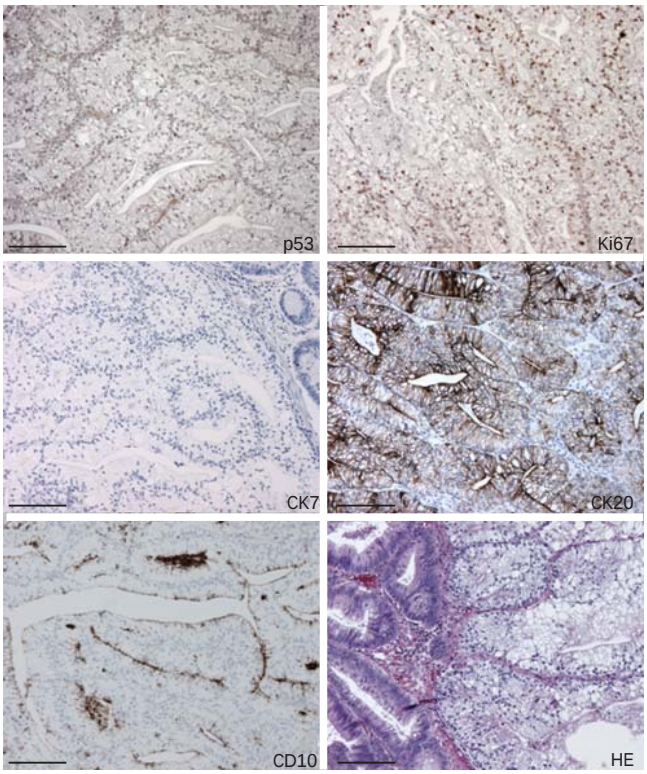


Figure 4 Ki67 is about 70% positive, p53 is positive, CK7 is negative, CK20 is positive, and CD10 is weakly positive on immunochemical staining. Each bar indicates 200 μm.

as well as a clear cell adenocarcinoma situated next to an adenoma. This supports the concept that the adenoma-carcinoma sequence of colorectal carcinogenesis is also valid for the clear cell adenocarcinoma. In our case, a clear cell adenocarcinoma is also presented adjacent to an adenoma.

Most reported cases of clear cell adenocarcinoma

Table 1 A review of primary clear cell adenoma and carcinomas of the colon

References	Age (yr)	Sex	Location	Treatment
Rubio[15]	68	M	Splenic flexure	Left hemicolectomy
Hellstrom and fisher[1]	67	M	Rectum	Abdominoperineal resection
Suzuki *et al*[24]	62	M	Descending colon	Polypectomy
Jewell *et al*[23]	26	F	Descending colon	Polypectomy
	75	M	38 cm from anus	Polypectomy
	56	F	Sigmoid colon	Sigmoidectomy
	61	F	Sigmoid colon	Polypectomy
Domoto *et al*[3]	54	M	Sigmoid colon	Polypectomy
	45	M	Transverse colon	Polypectomy
	44	M	Sigmoid colon	Polypectomy
McCluggage *et al*[9]	65	M	Rectosigmoid colon	Anterior resection
Braumann *et al*[25]	89	M	Transverse colon	Segmental resection
Current case	71	F	Sigmoid colon	Polypectomy

in the colon have been identified as a metastatic focus from other organs, including renal clear cell malignancies or those arising from an ovarian malignancy[14]. A rare combination of primary malignancies in both clear cell renal carcinoma and clear cell carcinoma of the colon has been reported in two patients[4]. In many cases, the differential diagnosis between advanced colonic clear cell adenocarcinoma and metastatic clear cell renal adenocarcinoma seems to be complicated. The similar histologic structure makes it difficult to distinguish between primary colonic clear cell adenocarcinoma and metastases[15]. Our case revealed no other tumors except for the original clear cell adenocarcinoma in the colon. In addition, the clear cell carcinoma occurring next to a normal colonic adenoma also supports the premise that it is the original (primary) tumor. We performed endoscopic polypectomy in this case, and pathological examination showed focal adenocarcinoma with clear cell change on the top of the polyp and common colonic adenoma on the body and base of the polyp. Vascular invasion and lymphatic involvement was not found. The cut margin was negative. As shown in Figure 2, this polypectomy was considered a curative treatment.

Cytokeratins (CK) are a family of at least 20 structural proteins found in the cytoskeletons of epithelial cells. Certain epithelia exhibit characteristic patterns of CK expression depending on the type, location, and differentiation of the epithelium. CK7, essentially, is not expressed in the normal epithelium of the gastrointestinal tract, whereas CK20 is expressed in the intestinal epithelium, the gastric foveolar epithelium, and the endocrine cells in the upper portions of the pyloric glands. On immunochemical staining, it was negative for CK7 and positive for CK20, also indicating that this tumor originated from the colonic mucosa, but not from the mullerian system.

To our knowledge, the proliferative activity among clear cells in adenomas has been rarely discussed. Immunohistochemical staining using antibodies against the Ki67 antigen is recognized to be a useful way to evaluate the proliferative activity of tumors. The increased Ki67 labeling index in the clear cell portion supported a high proliferative activity of the present tumor. The expression of the p53 protein is a well known and frequent abnormality in colorectal cancer, and it is involved in the progression of an adenoma to an adenocarcinoma. In our case, the p53 positivity tended to be higher in the clear cell portion than in the other portions, which supported the high malignant potential of the clear cells. On the other hand, a p53 overexpression has been described in 0%-40% of renal cell carcinomas[16-21] and ovarian clear cell carcinoma, and a p53 overexpression in 7% of other tumors[22]. The p53 immunostaining in this case also supports the evidence for a colonic origin of this colon tumor.

As shown in Table 1, prognosis was not described in most of the cases undergoing polypectomy. One patient was alive for 12 mo without any troubles[24]. This indicates that prognosis of clear cell carcinoma is still unclear. Further reports are necessary for elucidation of this tumor.

In conclusion, we herein reported a rare case of clear cell carcinoma in the colon which was successfully resected by an endoscopic polypectomy.

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CASE REPORT

Rapunzel syndrome: The unsuspected culprit

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Abstract

Trichobezoar is a rare intriguing disorder in which swallowed hairs accumulates in the stomach. Being indigestible and slippery, it could not be propulsed and becomes entrapped within the stomach. Large amounts can thus accumulate over the years forming a hair ball. Rapunzel syndrome is a variant where hair accumulation reaches the small gut and beyond in some cases. Although the syndrome has been known for many years, only 24 cases have been reported in the literature and the discovery of a new case is always surprising. In this report, we present two cases discovered within a period of three months. One of them was pregnant and had small bowel intussusception and perforation, a very rare combination. We hereby add two more cases to the literature. To our knowledge, this is the first report on two cases of Rapunzel syndrome, the diagnosis of which demands a high index of suspicion.

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Key words: Trichobezoar; Rapunzel syndrome; Intussusception

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INTRODUCTION

Reported in the 18th century[1], trichobezoar is a combination of "trich" meaning hair in Greek and "bezoar" meaning poison antidote in Arabic or Persian[2]. The habitual swallowing of hairs over the years leads to the formation of a hair ball in the stomach. Extension of hairs beyond the stomach into the small bowel in the form of a tail has been termed Rapunzel syndrome[3], after Rapunzel, the heroine of a German fairy tale[4].

CASE REPORT

Case 1

An 11-year-old female, was referred with an abdominal mass associated with epigastric pain, sense of fullness and vomiting after meals, for the previous 15 days. She was a poor school performer and had a history of pica (habitual ingestion of foreign bodies) and habitual thumb sucking. Her siblings, two brothers and two sisters, were all healthy and she had no family or social strains.

On examination, she was uncooperative and mildly dehydrated. Her temperature was 37℃, blood pressure 110/60 mmHg and pulse 90/min. The chest and heart examination were normal. Abdominal examination showed a firm mass about 8 cm × 10 cm in the epigastrium.

Her white blood cell count was 12800/μL (reference range 4000-10000), Hb 12.9 g/dL (reference range 12.1-15.1), platelet count 574000/μL (reference range 190000-405000). The rest of laboratory investigations showed serum proteins of 6.6 g/dL (reference range 6.4-8.2) and serum albumin of 2.7 g/dL (reference range 3.4-5.0). There was no electrolyte disturbance and liver functions, serum amylase and lipase were normal.

Computed axial tomography showed a mass with a hazy outline, filling the stomach. There were air pockets and the mass showed no contrast enhancement. So, the patient was prepared for surgery.

During surgery, a hair ball was found occupying the whole stomach (Figures 1A and 1B) and extending as far down as the jejunum. By gentle manipulation, it was extracted intact (Figure 2), and a gastric ulcer was also found (Figure 3).

After operation, the patient convalesced well, and her hospital stay was uneventful. She was discharged for follow-up in the Paediatric Psychiatry Clinic.

Case 2

A 19-year-old primigravida, at the end of the second trimester, was referred with upper abdominal pain, greenish vomiting, constipation and abdominal distension, for five days. Her history was positive for anorexia and failure to gain weight since childhood. There were no other medical

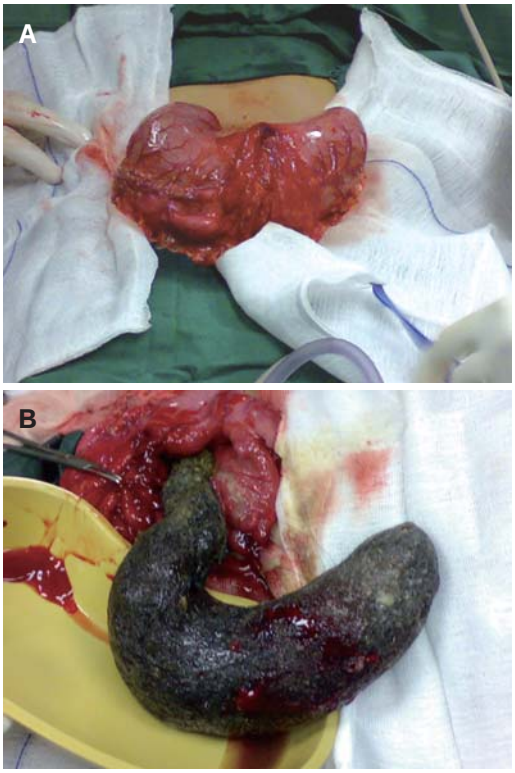


Figure 1 A hair ball occupying the whole stomach (**A**) and extending down into the jejunum (**B**).



Figure 2 Full extent of the hair ball extending into the jejunum.

or psychiatric problems.

On examination, the patient was cachectic and dehydrated, her blood pressure was 120/70 mmHg, pulse 105/min, and temperature 36.8℃. The chest and heart examination were normal.

Abdominal examination revealed a well defined, smooth and firm mass, 18 cm × 20 cm, occupying the epigastrium and almost both hypochondriae. The mass was resonant to percussion and moved freely with breathing.

Her white blood cell count was 5400/μL (reference range 4000-10 000), Hb 9.2 g/dL (reference range 12.1-15.1), platelet count 260 000/μL (reference range 190 000-405 000), Na 138 mEq/L (reference range 135-153), K 2.4 mEq/L (reference range 3.5-5.3). Her random blood sugar, renal values, liver function tests, serum lipase and amylase were within the normal range.

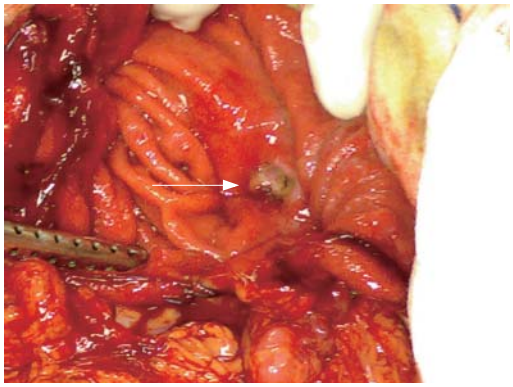


Figure 3 Ulcer in the body of stomach (arrow) caused by hair ball.

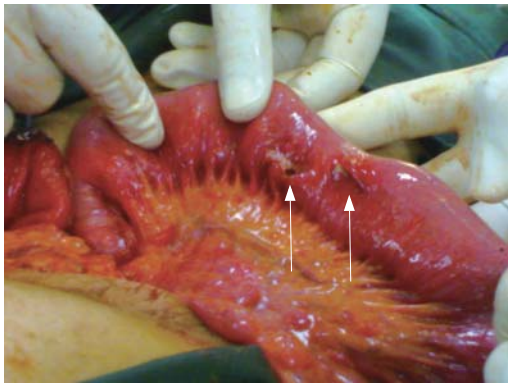


Figure 4 Two perforations at the mesenteric border of the proximal jejunum (arrows).

Abdominal ultrasound showed a large epigastric mass, with acoustic shadow suggestive of a calcified margin. Upper endoscopy disclosed the nature of the mass as a large hair ball occupying the whole stmoch, and the patient was posted for surgery.

At operation, a gastrotomy was done and the hair ball was found extending down to the upper jejunum, causing jejuno-jejunal intussusception. The intussusception was reduced and the hair ball was manipulated out. Two jejunal necrotic perforations were found at the mesenteric border necessitating resection (Figure 4). The gastrotomy was closed in two layers and the abdomen was closed with drainage.

The recovery period was complicated by foetal loss, wound infection and limited ability to receive enough oral feeds. A psychiatric evaluation diagnosed her having an adjustment disorder. The patient finally recovered well and was discharged for psychiatric follow-up.

DISCUSSION

Trichobezoar was first reported in 1779 by Baudamant[1]. The word is a combination of "trich" and "bezoar", with the former meaning hair in Greek, and the later meaning poison antidote in Arabic or Persian[2]. By necessity, trichotilomania (plucking of hairs) and trichophagia (swallowing of hairs) are the leading events.

Rapunzel syndrome, described first by Vaughan

and colleagues in 1968[3], is an extension of the gastric trichobezoar into the small gut in the form of a long tail. It is named after Rapunzel, the heroine of a German fairy tale by the Grimm brothers. She used to let her long golden hair tress down the tower where she was imprisoned, to allow her prince lover to climb up.

The syndrome has been rarely reported. In their report in 1999, Dalshaug and colleagues found only 11 cases[4], whereas in a recent search in 2007, only 24 cases were retrieved from the literature[5].

The reason why hair collects in the stomach is not fully understood. Debakey and Oschner suggested that hair entrapment in the gastric folds is the initiating event[6]. Due to its indigestibility, resilience and slippery nature, it becomes entrapped within the mucosal folds where it gets enmeshed, and acquires more hairs and thus a larger size.

The clinical picture ranges from abdominal pain, nausea, vomiting, early satiety and weight loss, to bleeding ulcer, intestinal obstruction and perforation. Intussusception has also been reported[4].

Although a psychiatric trouble is usually present[7], this is not always the case as the syndrome may affect healthy women[8]. In the first case reported here, the patient had a history of pica, which should be an alarming sign, mandating behavioural or psychiatric intervention. In the second case, although the patient denied any psychiatric troubles or social strains, on careful enquiry, her husband revealed that she had the habit of putting hair in her mouth while thinking.

Anaemia and hypoalbuminaemia associated with chronic gastritis usually go unnoticed until the case is brought to light by the onset of severer complications such as hemorrhage, enteric or pancreatic or biliary obstruction. The onset of perforation and peritonitis is largely responsible for an attendant mortality of about 30%[9]. In the second case, it was evident that intestinal perforation was a very recent onset as the patient showed no relevant signs on initial evaluation.

Trichobezoar affects young females almost exclusively[9] and it has been previously reported during pregnancy[10]. The presence of pregnancy may complicate the management, and foetal loss, as occurred in our second patient, is always a possibility. Provided there is no eminent danger, it might be wise to postpone intervention till the patient delivers.

As mentioned in the classic series of Debakey *et al*[6], an upper abdominal mass remains the commonest presenting sign. In our cases, an upper abdominal mass was easily visible due to its large size and the cachectic habitus of the patients.

Diagnostic modalities include US, CT scan and upper endoscopy. CT scan has a high accuracy rate, but the accuracy of US in such cases is not so high[12]. On CT scan, a well-circumscribed lesion, composed of concentric whorls of different densities with pockets of air enmeshed within it, appears in the region of stomach. Oral contrast fills the more peripheral interstices of the lesion and a thin band of contrast circumscribes it. Absence of significant post intravenous contrast enhancement precludes a neoplastic lesion[13]. In our second case, US failed to give the correct diagnosis, and as CT scan was not an option due to the presence of pregnancy, upper endoscopy was needed to reach the diagnosis. Endoscopy has also been reported to have a therapeutic potential[11]. It is probable that US is not the modality of choice in such conditions[11,12].

Laparoscopy may be successful in extracting the hair ball[10,14]. In our cases, this would not be an easy undertaking due to the young age of the first case and the presence of intussusception and pregnancy in the second. Other minimally invasive modalities like extracorporeal lithotripter, endoscopic lithotripter, and laser fragmentation[9] are emerging. Their role and success rates need to be defined and open surgery remains the gold standard.

In conclusion, surgeons, physicians, and radiologists should consider trichobezoar in the differential diagnosis of gastrointestinal obstruction in young females, especially in the presence of an upper abdominal mass. Timely diagnosis and treatment are of utmost importance to avoid a possible fatal outcome. As recurrences are known, each patient should have a proper psychiatric evaluation and follow-up.

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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]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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]

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]

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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

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Conference paper

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Electronic journal (list all authors)

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/eid.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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EDITORIAL

Role of symptoms in diagnosis and outcome of gastric cancer

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Abstract

Gastric cancer is one of the most common cancers and the second most common cause of cancer deaths worldwide. Apart from Japan, where screening programmes have resulted in early diagnosis in asymptomatic patients, in most countries the diagnosis of gastric cancers is invariably made on account on dyspeptic and alarm symptoms, which may also be of prognostic significance when reported by the patient at diagnosis. However, their use as selection criteria for endoscopy seems to be inconsistent since alarm symptoms are not sufficiently sensitive to detect malignancies. In fact, the overall prevalence of these symptoms in dyspeptic patients is high, while the prevalence of gastro-intestinal cancer is very low. Moreover, symptoms of early stage cancer may be indistinguishable from those of benign dyspepsia, while the presence of alarm symptoms may imply an advanced and often inoperable disease. The features of dyspeptic and alarm symptoms may reflect the pathology of the tumour and be of prognostic value in suggesting site, stage and aggressiveness of cancer. Alarm symptoms in gastric cancer are independently related to survival and an increased number, as well as specific alarm symptoms, are closely correlated to the risk of death. Dysphagia, weight loss and a palpable abdominal mass appear to be major independent prognostic factors in gastric cancer, while gastro-intestinal bleeding, vomiting and also duration of symptoms, do not seem to have a relevant prognostic impact on survival in gastric cancer.

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Key words: Gastric cancer; Symptoms; Diagnosis; Survival; Cancer stage; Alarm symptoms; Dyspepsia

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INTRODUCTION

Although the incidence of gastric cancer is decreasing in many countries, it remains one of the most common cancers and the second most common cause of cancer deaths worldwide[1]. Since the early stage of the disease is often asymptomatic, diagnosis is frequently made at an advanced stage of the disease, characterized by poor prognosis with a reported 5-year survival rate of less than 30% in most series.

Unlike Japan, where the screening programme resulted in a 5-year survival rate of 90%, in Western countries, despite the introduction of open-access gastroscopy, delays in diagnosis still seem to be common and virtually affect the stage of gastric cancer at diagnosis as well as outcome of patients. In fact, in Western countries, where a screening programme is not feasible[2], diagnosis of gastric cancer inevitably relies on symptoms reported by patients. Therefore, the increased awareness of symptoms on the part the patients, as well as correct interpretation of the symptoms and prompt referral for investigation, could reduce the diagnostic delay and, theoretically, improve survival.

To date, perception of symptoms and reasons for seeking, or not, consultation have been more extensively investigated in functional gastro-intestinal disorders than in organic diseases[3,4] and, in several studies, the interpretation and analysis of dyspeptic symptoms seemed to be focused more on reducing the endoscopic workload than on improving the detection of gastro-intestinal malignancy. Finally, the few studies that have analysed the influence of diagnostic delay, namely the symptom-to-diagnosis interval, on survival in gastric cancer have shown disappointing results.

The present review, while focusing attention on an analysis of the literature dealing with the symptoms in diagnosing and suggesting prognosis of gastric cancer, aims to elicit insights into some clinical (unexplored or forgotten) aspects of dyspeptic symptoms and gastric cancer.

IMPORTANCE OF SYMPTOMS IN DIAGNOSIS OF GASTRIC CANCER

Dyspeptic symptoms and also the alarm symptoms that

usually identify dyspeptic patients at higher risk of organic dyspepsia are common not only in patients consulting the general practitioner but also in the general population[5]. Gastro-oesophageal reflux disease, peptic ulcer disease, and functional dyspepsia represent the most frequent causes of dyspeptic symptoms. Only in a few cases are dyspeptic symptoms caused by gastro-oesophageal malignancy[6].

Upper gastro-intestinal endoscopy is usually performed to study patients with dyspeptic symptoms. Endoscopy is, however, expensive, troublesome to the patient, and, in more than 50% of endoscopic examinations, no organic cause can be found[7]. Therefore, non-invasive treatment strategies such as *H pylori* test-and-treat or empirical treatment with acid inhibitory drugs, i.e., proton pump inhibitors and H_2-antagonists, have been proposed and it has become evident that these strategies are at least as effective as upper gastro-intestinal endoscopy followed by targeted treatment[7-10].

However, the main concern, in the application of empirical treatments of dyspepsia are the possibility of missing gastric cancer or of delaying the time to diagnosis. An accurate selection of patients with a higher risk of gastro-oesophageal cancer, to be immediately submitted to endoscopy, is thus very important. Alarm symptoms such as weight loss, dysphagia, signs and symptoms of upper gastro-intestinal bleeding, anaemia, and persistent vomiting, are likely to be more frequently associated with upper gastro-intestinal malignancies, and most guidelines recommend immediate endoscopy in all patients presenting these symptoms[11-13].

The evidence supporting the use of alarm symptoms as selection criteria for endoscopy is, however, inconsistent since, on the one hand, alarm symptoms are not sufficiently sensitive to detect malignancies and, on the other, the overall prevalence of alarm symptoms, in a population of dyspeptic patients is high, while the prevalence of gastro-intestinal cancer is very low.

The studies, so far, reporting a high prevalence of alarm symptoms in gastro-intestinal malignancies are mainly retrospective and, according to these studies, up to 90% of patients with gastro-oesophageal malignancies present alarm symptoms at the time of endoscopy[14-16]. Large prospective cohort studies have achieved less significant results. For example, Meineche-Schmidt[17] showed that, in a large cohort of primary care patients with dyspeptic symptoms, the majority of patients who developed gastric/oesophageal cancer did not present with any alarm symptoms, while Lieberman[18], who studied a large database of endoscopy reports, showed that only 56% of patients with gastric/oesophageal cancer had alarm symptoms.

This discrepancy in results between the different studies may be explained by the fact that, in retrospective studies, patients may have had more advanced cancers which are less often asymptomatic[19] or that the retrospective analysis may have over-estimated the prevalence of alarm symptoms before endoscopy.

Due to the low prevalence of gastric and oesophageal cancer in the population, studies on larger series are needed. A recent investigation by Janssen, which appeared as an abstract[20], combined individual patient data from 7 prospective studies thus referring to more than 13 000 dyspeptic patients undergoing endoscopy. This investigation, which likely offers a more reliable picture of the problem, reported a prevalence of alarm symptoms of 30% (3927/13 377) and of 62% (103/165) in patients with gastro-intestinal cancer. This resulted in a sensitivity of 62.4%, a specificity of 70.5%, a positive predictive value of 2.6%, and a negative predictive value of 99.3% when the parameter "having any alarm symptom" was used to diagnose the presence of cancer. Interestingly, none of each individual alarm symptom was able to identify more than 30% of patients with gastric or oesophageal cancer. Moreover, the prevalence of alarm symptoms did not differ between young (< 50 years) and elderly (> 50 years) patients (11/19, 57.9% *vs* 92/146, 63%).

The role of each individual alarm symptom for predicting cancer in patients referred to a "rapid access upper gastro-intestinal cancer" service was evaluated by Kapoor[21]. In that study, involving, prospectively, 1785 patients referred for urgent endoscopy for suspected organic disease (which derived either from the occurrence of alarm symptoms or the presence of non-responsive, uncomplicated dyspepsia), occurrence of gastro-intestinal malignancies and benign organic disease was 3.8% and 12.8%, respectively. Dysphagia and weight loss were the only symptoms found to represent significant predictive factors for cancer [Odds Ratio (OR) 3.1 and 2.6, respectively]; age > 55 years was also a predictor of cancer (OR 9.5) but, uncomplicated dyspepsia, irrespective of age, was actually found to be a negative predictor of cancer (OR 0.1). From these data the authors developed a model in which, in dyspeptic patients, dysphagia and weight loss would represent criteria to perform endoscopy at any age, while age > 55 years would represent an indication for immediate endoscopy, only in the presence of an alarm symptom.

To improve the diagnosis of gastro-intestinal cancer, age is considered an important factor. There is a gradual increase in the risk of gastric and oesophageal cancer with age, but, unfortunately, a clear cut-off is difficult to define. Although, in the vast majority of the populations studied, almost 90% of patients with cancer are > 50 years, albeit the age distribution of upper gastro-intestinal cancer may vary; indeed, in Poland, Boldys[22] showed that, in a high-risk tertiary care population, as many as 24% of patients with gastric cancer were < 45 years.

Use of the age criteria to select dyspeptic patients to investigate with endoscopy is considered safe by most authors, provided the age cut-off has been determined using data from the local population[23-25]. Male sex is also significantly associated with gastro-intestinal cancer and, according to some authors; the age threshold for endoscopy should be lowered in males to decrease the risk of missing cancer, and could be safely increased in females without affecting outcomes[25].

Approximately 3%-4% of the population, in industrialized countries, consult their general practitioner with upper gastro-intestinal symptoms each year, of which over 10% will have alarm symptoms[26]. The symptoms of early stage cancer may be indistinguishable from those of benign dyspepsia, while the presence of established

alarm symptoms may imply advanced inoperable disease. This represents a dilemma in terms of defining criteria and priorities for investigating upper gastro-intestinal symptoms, particularly in healthcare systems where the potential demand for endoscopy may exceed the level of provision.

Current guidelines, based on the rising incidence of malignancy with age, suggest that patients with uncomplicated dyspepsia be investigated, only if older than a cut-off age[12,13], but the concern of missing cancer in young dyspeptics may be overcome only by investigating every patient with recent-onset dyspeptic symptoms regardless of age. The Canadian (CADET) study, which examined 1021 patients referred from primary care units, without alarm symptoms, identified only 2 cancers[27]. Other studies have demonstrated that the endoscopic yield of cancer, in simple dyspepsia, is low and only very few of the identified cancers are resectable[15,28].

We do not know whether the incidence of malignancy in patients with uncomplicated dyspepsia is different from that in the non-dyspeptic population; but we do know that early gastro-intestinal cancers are asymptomatic and are usually diagnosed by chance in patients undergoing endoscopy for dyspeptic symptoms secondary to benign or functional conditions. The impact of delaying the diagnosis of gastric cancer while giving a short course of empirical treatment is unknown, but, probably, a strategy of initial non-invasive management is unlikely to significantly affect the outcome, in the majority of young patients with benign dyspepsia provided the physician promptly arranges endoscopy where appropriate, i.e., in the event of lack of response to empirical therapy[29].

PROGNOSTIC RELEVANCE OF SYMPTOMS IN GASTRIC CANCER

The prognosis of gastric cancer has improved, in recent years, at least in Eastern countries where the 5-year survival rate is 50%-60%[30-32]. On the contrary, survival rates in Western series are still dismal, ranging between 8% and 26%[33-37]. One of the main reasons for this discrepancy is the efficacy of screening programmes in Japan, where the high incidence and awareness of the condition, and ready access to endoscopy have led to cancers usually being diagnosed in asymptomatic patients or in patients with minor dyspeptic symptoms[38]. In Europe and Western countries, where a screening programme is not feasible, the diagnosis of gastric cancers is invariably made on account of symptoms.

Indeed, the onset, duration and features of dyspeptic and alarm symptoms may suggest the diagnosis of gastric cancer, but they also reflect pathological features of the tumour and, therefore, have some prognostic value.

To date, the survival rates of gastric cancer, as well as the differences in survival rates observed between Eastern and Western case series, have been mainly attributed to the stage of the tumour, namely the depth of gastric wall invasion and status of lymph node and metastasis. Reports in the literature investigating the prognostic factors in gastric cancer, have focused mainly on the relevance of tumour-related features (such as, tumour node metastasis (TNM) classification, histological type, tumour appearance, site and size) or treatment-related factors (extent of gastric resection and lymphadenectomy and post-operative treatment) other than on patient-related clinical features.

However, since the eighties and more consistently in recent years, the prognostic role of symptoms, particularly alarm symptoms, in gastric cancer survival, has been investigated.

Symptoms and cancer stage

The alarm symptoms accompanying dyspeptic symptoms do not only have a diagnostic role in indicating the possible presence of gastric cancer, but when referred to, at diagnosis, in gastric cancer patients, may suggest the site, stage and aggressiveness of cancer and usually indicate poor prognosis.

In a retrospective study on a series of 92 young gastric cancer patients, we have evaluated the relationship of alarm symptoms, such as dysphagia, anorexia, weight loss, gastro-intestinal bleeding and vomiting with pathology (intestinal or diffuse) and site of cancer and stage, according to the TNM classification. Patients presenting with alarm symptoms were comparable to those with uncomplicated dyspepsia as far as concerns age, sex and pathology of gastric cancer, but more frequently showed a proximal site and more advanced TNM stage[19]. These findings have been confirmed by Stephens, who also showed that the number of alarm symptoms, at presentation, correlated with the stage of the tumour, in that patients with the greatest number of alarm symptoms presented with the most advanced disease[39] and by Bowrey, in a more recent study, in which approximately 50% of the patients with alarm symptoms had stage Ⅳ disease[40].

Indeed, the relationship between symptoms and stage of cancer is conceivable if we consider the profile of symptoms in early gastric cancer which are not unlike those of benign gastric ulcer rather than advanced cancer. Epigastric pain and dyspepsia are very frequently present, whilst alarm symptoms occur in only a minority of patients. For instance, weight loss occurs in less than 40% of patients, in early gastric cancer, while it is a common feature of advanced gastric cancer[41]. The reasons triggering benign or malignant symptoms in gastric cancer, and whether benign may convert to malignant symptoms over time, deserve further investigation.

Specific symptoms and outcome of gastric cancer

Since symptoms correlate well with cancer stage at diagnosis, they are likely also of prognostic value. Indeed, several studies have assessed the prognostic value of specific alarm symptoms in gastric cancer, demonstrating that these may be independently related to the survival of patients with gastric cancer and that, an increased number of alarm symptoms and specific symptoms are closely correlated to the risk of death. Studies that evaluated the impact of alarm symptoms on gastric cancer survival showed that the presence of at least one alarm symptom might reduce the 5-year survival rate by an average of 26%[19,39,40]. We have also shown that the

risk of death is nearly threefold in patients with at least one alarm symptom compared with that in patients with uncomplicated dyspepsia[19]. Moreover, Stephens and Bowrey reported that the median survival for patients with alarm symptoms, from presentation to death, ranged from 7 to 11 mo *vs* 24 to 39 mo for patients without alarm symptoms[39,40].

These dismal prognostic data in gastric cancer patients with alarm symptoms are not completely new. Earlier studies and recent confirmation have shown that any symptom may have a significant and independent prognostic role in gastric cancer patients and that specific symptoms may have a particularly greater prognostic impact.

It is difficult to analyse, compare and describe the prognostic role of each alarm symptom reported in the literature on account of the different definition criteria of symptoms, retrospective collection of data in most studies, geographic and time differences in treatment-related prognostic factors, and different age of patients enrolled in the studies. However, of the alarm symptoms most widely considered, namely dysphagia, weight loss, palpable abdominal mass, gastro-intestinal bleeding, anaemia, persistent and continuous vomiting, the data published so far have consistently identified dysphagia, weight loss and palpable abdominal mass as the most relevant and independent prognostic factors in gastric cancer.

Weight loss has been considered among the potential prognostic factors for gastric cancer by at least 13 studies so far, and in 10 of these it resulted significantly and independently related to the fatal outcome[19,39,40,42-51]. It is not possible to calculate life expectancy in gastric cancer patients presenting with weight loss. However, the 5-year survival rate ranged from 13.5% to 31%[39,44] and the mean survival reported was less than 1.2 years[46,40]. Indeed, the prognostic relevance of this symptom is well known. It correlates with malnutrition and impaired immune response. Weight loss does not merely increase post-operative complications, but also reduces the response and increases the toxicity to chemotherapy[45,48,52]. Indeed, it has been shown that 10 d of pre-operative total parenteral nutrition (TPN), which is continued post-operatively, reduces the complication rate and prevents mortality in severely malnourished patients with gastro-intestinal cancer[53].

As far as concerns dysphagia, 5 studies evaluated the prognostic relevance of this symptom in gastric cancer, all of which showed that is was significantly associated with poor outcome[19,39,40,44,46]. Indeed, the prognosis of patients presenting this symptom seems to be even worst than that reported for weight loss, with a 5-year survival rate of 6%-7%[39,44], a mean survival < 10 mo[40,46] and risk of death > 7 times compared to that of patients without alarm symptoms[19]. Dysphagia usually identifies tumours of the cardia and proximal part of the stomach with prognosis being worse than in other locations for gastric cancer. Moreover, it is usually associated also with malnutrition and weight loss.

Another alarm symptom which is invariably associated with poor prognosis is the presence of a palpable abdominal mass. Eight studies have assessed the prognostic value of this sing[39,40,44-47,54] and all but one found a strong association with a very low 5-year survival rate (0%-20%)[39,44,54] and also with a very short life span (< 4 mo)[40,46].

The prognostic role of vomiting and gastro-intestinal bleeding is more controversial. As far as concerns vomiting, as an alarm symptom, 2 out of 4 studies[19,39,40,46] evaluated the prognostic significance of this symptom and showed that is was correlated with brief survival, namely a median survival of 9 mo, in one study[40], and a 5-year survival of 14%, in the other[39]. Indeed, this symptom implies a certain degree of subjective interpretation and it may be difficult to retrospectively consider it as an alarm symptom, if persistent and continuing, or as a 'simple' dyspeptic symptom if occasional or of mild degree.

Gastro-intestinal bleeding has been regarded as a prognostic symptom in 4 studies[19,39,44,47] but in none showed any significant impact on outcome, probably because haemorrhage may appear even in earlier phases of the tumour and may not necessarily be related to the stage. In none of the studies investigating the role of specific dyspeptic symptoms, such as epigastric pain or abdominal discomfort, were these significantly related to survival.

Duration of symptoms and outcome of gastric cancer

The stage of gastric cancer, at diagnosis, has been regarded as the most important prognostic factor for survival. We have shown that it is correlated with the presence of specific symptoms. It has been argued that prolonged duration of symptoms, or - in other words - delay in diagnosis, could result in missing a gastric cancer at a hypothetically curable stage and, therefore, with a negative effect on survival[55].

For this reason, several studies have assessed the impact of dyspeptic versus alarm symptoms on diagnostic delay, and of symptoms-to-diagnosis time interval on survival in gastric cancer. All studies showed, as expected, that delay in diagnosis was longer for patients without alarm symptoms compared to that of patients with alarm symptoms[19,39,40]. However, all these studies also showed that, despite the diagnostic delay, survival rate of patients with gastric cancer who lacked alarm symptoms, at the time of diagnosis, was better than that in patients with alarm symptoms at presentation. Paradoxically and more important, patients with uncomplicated dyspepsia with a delay more than 6 mo showed better survival rates[19,39,55-59].

Indeed, the duration of symptoms has been considered as a relevant prognostic factor in several studies. At least 14 studies have evaluated this prognostic aspect but all showed that duration of symptoms was not related to poor outcome[19,32,43-46,60-67]. It would have seemed obvious that the sooner a patient is treated for cancer, the better the prognosis will be. On the contrary, almost all studies published, so far, have shown that delay in diagnosis is not a relevant prognostic factor and more than one study demonstrated that long duration of symptoms is associated with the earlier the tumours are diagnosed, longer the survival[19,46,59,60].

Other studies have shown that gastric cancer patients with alarm symptoms have a poor prognosis despite a much shorter symptom-to-diagnosis time interval

compared to patients without alarm symptoms, providing indirect evidence that the relationship between duration of symptoms and prognosis is less important than the characteristics of the symptoms at presentation.

One possible explanation for this is that mild or dyspeptic symptoms, that do not alarm the patient or his/her doctor, might be associated with a cancer disease presenting a less aggressive behaviour and slower progress[68]. But this remains to be demonstrated, to date. Another possible explanation is that the delay in diagnosis in patients without alarm symptoms may, instead, be due to the long duration of dyspeptic symptoms, the onset of which had not been related to gastric cancer. In this case, less symptomatic patients tend to have a less severe/advanced disease.

However, it is noteworthy that this inverse relationship between delay in diagnosis and survival has been consistently observed in several studies in various parts of the world. The potential practical implication for this is that a diagnostic delay of 6 mo since presentation of symptoms could be an acceptable time to evaluate the outcome of treatment in patients presenting with uninvestigated, uncomplicated, dyspepsia, without affecting survival if gastric cancer is already present. This has also been demonstrated already in two clinical studies in which anti-secretory drug treatment delayed the diagnosis of upper gastro-intestinal adenocarcinoma without affecting outcome, in most patients[29], and by another where the outcome of patients in whom the cancer was missed, at a previous gastroscopy, showed a similar outcome to that of patients with prompt correct diagnosis[69].

However, it cannot be excluded that improving the diagnosis of gastric cancer, when it presents without alarm or severe symptoms, may result in better prognosis[70]. To date, in fact, the behaviour of symptoms, in gastric cancer, is still poorly understood. Gastric cancer patients with alarm symptoms, at diagnosis, may have complained of uncomplicated dyspepsia at the onset of their symptoms, and developed alarm symptoms later. We do not know whether these patients differ from those presenting with alarm symptoms, as far as concerns delay of diagnosis, features and stage of gastric cancer. Further prospective studies are, therefore, needed not only to evaluate this aspect but would undoubtedly contribute to reaching a more accurate evaluation of symptoms and their impact on prognosis. Symptoms may vary from the onset to the diagnosis and referral of symptoms may depend on social and cultural factors[71]. A supportive study, in this direction carried out by Meineche-Schmidt and Jørgensen[17] in a primary care setting revealed that the majority of patients in whom upper gastro-intestinal cancer developed did not have alarm symptoms at their initial primary care consultation. It thus follows that the diagnosis of upper gastro-intestinal cancer, in most patients, is delayed until alarm symptoms occur, with inevitable consequences in terms of prognosis.

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OBSERVER

Adult autoimmune enteropathy

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Abstract

Recent reports have suggested that autoimmune enteropathy involving the small bowel may occur in adults as well as in children. Apparently, the endoscopic and histological changes are similar to celiac disease before treatment, but these are not altered by any form of dietary restriction, including a gluten-free diet. As in celiac disease, histologic changes in gastric and colonic biopsies have also been recorded. Anti-enterocyte antibodies detected with immunofluorescent methods have been reported by a few laboratories, but these antibodies appear not to be specific and may simply represent epiphenomena. A widely available, reproducible and quantitative anti-enterocyte antibody assay is needed that could be applied in small bowel disorders that have the histological appearance of celiac disease, but fail to respond to a gluten-free diet.

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Key words: Celiac disease; Autoimmune enteropathy; Anti-enterocyte antibodies; Anti-goblet cell antibodies; Antibodies in celiac disease

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COMPONENTS OF DIAGNOSIS

A number of reports in adults[1-7], similar to prior publications in children[8-10], have described an entity, termed autoimmune enteropathy (AIE), that appears to involve mainly, but not exclusively, the small intestine[4,7]. Some of the histologic changes, at least in adults, have been reported to permit pathological distinction from other disorders characterized by severe small bowel mucosal architectural abnormalities, specifically untreated celiac disease (Table 1). Specifically, reduced numbers of intra-epithelial lymphocytes, especially in surface epithelium, may be evident in AIE compared to adult celiac disease[7]. In AIE, the diarrhea fails to respond to any form of dietary restriction, including a gluten-free diet[8]. Circulating anti-enterocyte and/or anti-goblet cell antibodies are generally present in diagnosed AIE[7], but their precise significance in the pathogenesis of this disorder is not clear. It has been postulated elsewhere that these antibodies likely only represent epiphenomena since these appear after the onset of mucosal damage and then disappear following treatment, but before return to normal mucosa[11]. In some, pathological changes in gastric and colonic biopsies have also been recorded[7].

The antibodies are reported to appear as a linear pattern along the apex or brush border of the enterocytes, sometimes with an extension along the baso-lateral membrane[10]. Usually, the antibodies are IgG in type, sometimes complement-fixing, while in some, IgM and IgA antibodies occur[7,10]. These anti-enterocyte antibodies do not appear in celiac disease, Crohn's disease or ulcerative colitis[10]. Antibodies may also react with mucus in goblet cells, but these seem to be even less specific than anti-enterocyte antibodies[10]. For example, antibodies to goblet cells have been detected in chronic inflammatory bowel disease patients and their first-degree relatives[12].

Other co-existent autoimmune disorders as well as antibodies of different types have been observed in AIE. Indeed, even antibodies to tissue transglutaminase (tTG) have been described in over 30% of the largest series of reported AIE patients[7]. However, the presence of an associated autoimmune disorder is not a truly distinctive or differentiating clinical feature as autoimmune disorders have been commonly reported in several small bowel disorders, particularly in celiac disease, regardless of duration of gluten exposure[13-16].

DIAGNOSTIC DIFFICULTIES AND THE FUTURE

Most clinicians are likely to encounter significant difficulties in securing a definite diagnosis of AIE, because its precise differentiation from other small bowel disorders may be very difficult, at least based on current suggested criteria for diagnosis of adult AIE[7]. A number of factors have come into play. First, dietary compliance, even in an

Table 1 Comparison of celiac disease and autoimmune enteropathy in adults

	Celiac disease	Autoimmune enteropathy
Diarrhea	Responds to gluten-free diet	No response to diet therapy
Endoscopic and microscopic changes	Not specific, responds to gluten-free diet	Not specific, similar to celiac disease (pre-gluten-free diet)
Gastric and colon microscopic changes	May be abnormal	May be abnormal
Ages reported	All ages	All ages, mainly children
Autoimmune phenomena	Present	Present
TTG antibodies	Usually present	Present in 30% or more
Prognosis	Good with gluten-free diet	Unknown, anecdotal results

adult, may be exceedingly difficult to define. If dietary indiscretion is intentional or unavoidable, it may be very obvious. However, most experienced clinicians know that it may be very difficult to confirm that a specific diet has been continuously followed for prolonged periods (eg., gluten-free diet in celiac disease). Alternative "markers" have been suggested. For example, reduced antibodies to tTG in celiac disease have been suggested by some to provide a marker of compliance to a gluten-free diet, but others dispute this claim[17-19]. Second, failure to resolve symptoms with dietary restriction (eg., gluten) should not necessarily translate into a diagnosis of a persistent or refractory disorder. Other concomitant causes for symptoms, including functional disorders, may co-exist. Third, evidence for persistent histopathological changes in spite of dietary restriction may be more reflective of an inadequate duration of dietary restriction in order to detect a response or an inadequate method of assessment. In celiac disease, for example, histological evidence of normalization of pathological changes may require months or years, and usually occurs first in the most distal sites of involvement. Only later, sometimes much later, can a definite histopathological response to a gluten-free diet be documented in the most proximal small bowel (where endoscopic biopsies are generally taken). Fourth, only a limited number of laboratories claim to be able to detect anti-enterocyte antibodies, even though required methodology is straightforward, i.e., immunofluorescence microscopy. While anti-enterocyte antibodies are apparently not present in celiac disease[10], it appears that some of the histopathologic features of adult AIE may co-exist with those of celiac disease[7], further confusing the clinical picture.

In addition, these anti-enterocyte antibodies are not specific for AIE being reported in HIV-associated immunodeficiency syndromes without intestinal symptoms[20]. As noted earlier, anti-goblet cell antibodies which may be even less specific have been described in inflammatory bowel disease patients and their relatives[12]. Finally, and most importantly, immunofluorescent methods for serum antibody detection are very subjective, and observer-dependent. Use of immunofluorescent methods for screening (eg. anti-endomysial antibodies for celiac disease) are often based on "in-house" assays and may be difficult to reproduce between different laboratories, even if the same sources of sample material are used.

Further studies are needed to determine if AIE in adults is truly a separable clinical entity that may be amenable to other treatments. The most critical element needed is a widely available, reproducible, sensitive and specific assay for anti-enterocyte antibodies that might permit separation of AIE from other small bowel disorders and be an important addition to the investigative methods currently available for persistent diarrhea disorders in adults. To date, different therapeutic options have been used in an anecdotal fashion in adults diagnosed with AIE[7], but a conclusive approach to treatment still requires further evaluation.

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REVIEW

Current status and future strategies of cytoreductive surgery plus intraperitoneal hyperthermic chemotherapy for peritoneal carcinomatosis

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Abstract

This article is to offer a concise review on the use of cytoreductive surgery (CRS) plus intraperitoneal hyperthermic chemotherapy (IPHC) for the treatment of peritoneal carcinomatosis (PC). Traditionally, PC was treated with systemic chemotherapy alone with very poor response and a median survival of less than 6 mo. With the establishment of several phase Ⅱ studies, a new trend has been developed toward the use of CRS plus IPHC as a standard method for treating selected patients with PC, in whom sufficient cytoreduction could be achieved. In spite of the need for more high quality phase Ⅲ studies, there is now a consensus among many surgical oncology experts throughout the world about the use of this new treatment strategy as standard care for colorectal cancer patients with PC. This review summarizes the current status and possible progress in future.

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Key words: Peritoneal carcinomatosis; Cytoreductive surgery; Intraperitoneal hyperthermic chemotherapy; Gastric cancer; Colorectal cancer; Ovarian cancer; Peritoneal mesothelioma

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INTRODUCTION

The loco-regional progression of gastrointestinal and gynecological cancers usually result in peritoneal carcinomatosis (PC), which is characterized by the presence of tumor nodules of various size, number and distribution on the peritoneal surface, with very poor prognosis and a median survival of less than 6 mo[1-3]. The most widely accepted therapies for such PC are systemic chemotherapy, best support care and palliative treatment, without any hope of cure. Moreover, surgery alone can only remove the bulky visible tumor nodules. For the micrometastases, invisible free cancer cells and those tumor masses not suitable for resection, surgery can not achieve any effect. Therefore, neither surgery nor chemotherapy alone can make obvious positive impact on the survival and quality of life in patients with PC. In order to tackle this difficult problem, a new treatment modality called cytoreductive surgery (CRS) plus intraoperative peritoneal hyperthermic chemotherapy (IPHC) has been developed over the past two decades, which has the advantages of surgery to reduce the visible tumor burden and regional hyperthermic chemotherapy to eradicate micrometastses and free cancer cells[4]. Over the past decade, this treatment modality has gained increasingly wide acceptance in clinical practice and in some cancer centers in Europe, America, Japan and Australia, and has been adopted as the treatment of choice for PC from gastrointestinal tract or pelvic malignancies. This paper summarizes the biological basis, the indications and contraindications, the techniques, the efficacy and safety issues and future directions of this new treatment.

INCIDENCE, MORBIDITY AND MORTALITY OF PC

PC is a direct consequence of loco-regional progression

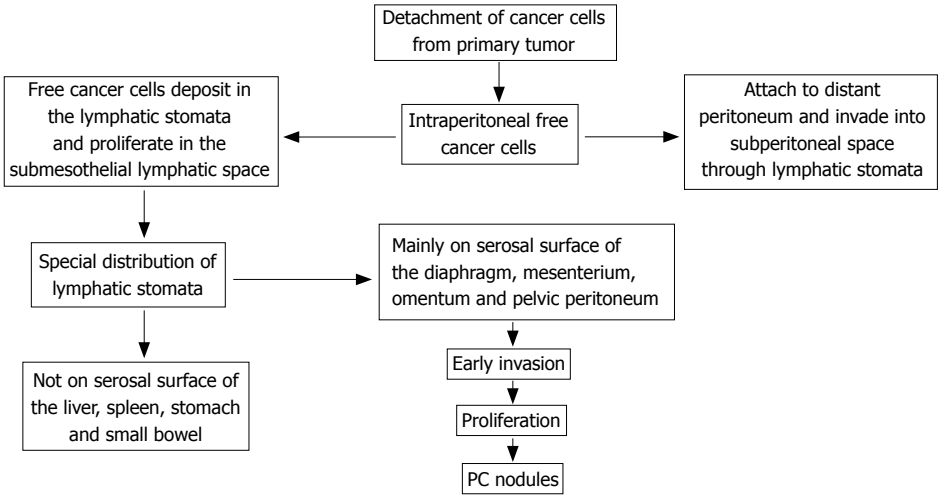


Figure 1 Mechanism of PC development.

of gastrointestinal and gynecological cancers including gastric cancer (GC), colorectal cancer (CRC), ovarian cancer (OC), appendiceal cancer and malignant peritoneal mesothelioma.

For GC, 10%-20% of patients being explored for potentially curative resection are found to have peritoneal seeding at the time of abdominal exploration. This occurs most frequently in patients with signet ring cell type of GC as opposed to intestinal type pathology[5]. Furthermore, peritoneal recurrence develops in 60% of patients with T3 or T4 tumor after curative resection[6]. In T3 or T4 tumor, invisible micrometastases are already present in the peritoneal cavity at the time of curative resection, and peritoneal recurrence is the only site of the first recurrence in 40%-60% of patients[7-9]. Therefore, peritoneal dissemination alone usually results in death of 20%-40% of patients with GC[10].

For CRC, peritoneal seeding of cancer cells leading to PC is rather common. It is estimated that, at the time of diagnosis, the peritoneal surface is already involved in 8%-15% of CRC cases, and that initial recurrence in the peritoneum occurs in up to 50% of patients after curative surgery[11-14]. In approximately 25% of patients with recurrent CRC, the peritoneal cavity seems to be the only metastatic site of the disease, even after a detailed diagnostic workup of the liver and lungs[15].

OC is another malignancy prone to peritoneal metastasis. About 50%-75% of women with OC will develop persistent or recurrent disease with a long survival rate in only 25% patients[16,17]. In about 82% of the cases, the recurrence is within the peritoneal cavity while in 12% it occurs in the retroperitoneal lymph nodes[18-20]. For early stage OC, the median recurrence time ranged between 11 mo and 29 mo with much more involvement of the pelvis or abdomen than retroperitoneal lymph nodes[21-28]. For advanced OC, if the initial treatment achieves meaningful clinical response, the median recurrence time ranges from 18 mo to 24 mo, again with peritoneal metastasis as the most common site[16,19,29-34].

MECHANISMS OF PC DEVELOPMENT

PC is formed through a multi-step process (Figure 1): (1) detachment of cancer cells from primary tumor. The origin of PC in GC, for example, is considered to be the intraperitoneal free cancer cells, which are exfoliated from the serosal surface of the primary tumor; (2) intraperitoneal free cancer cells attach to the distant peritoneum, and invade into the subperitoneal space; (3) invasion into subperitoneal space; and (4) proliferation with vascular neogenesis[35,36].

Recent studies have revealed special anatomical basis for peritoneal metastasis. Yonemura *et al*[9,37] found special peritoneal lymphatic orifices, which are referred to as the lymphatic stomata and connect with subperitoneal lymphatic channel and milky spots. Milky spots are the minute organelles which contain lymphatic vessels, lymphocytes, and peritoneal macrophages. Milky spots distribute mainly on the greater omentum and pelvic peritoneum. Intraperitoneal free cancer cells specifically deposit in the lymphatic stomata and proliferate in the submesothelial lymphatic space. The lymphatic stomata showed special distribution on the peritoneal surface. Numerous stomata are detected on the undersurface of the diaphragm, small bowl mesentery, greater omentum, appendix epiploicae of the large bowel and the pelvic peritoneum. In contrast, there are no lymphatic stomata on the liver capsule, the surface of the spleen and the serosal surface of the small bowel and stomach. Accordingly, the serosal surface of these organs is involved only at the late phase of peritoneal dissemination.

CURRENT TREATMENT STRATEGIES AGAINST PC

For a long time, PC has been considered as a preterminal condition and treated with systemic chemotherapy alone with poor outcome; and the role of surgery is just palliative to relieve intestinal obstruction. But at least 1 phase Ⅲ and many phase Ⅱ studies have shown that the use of CRS with IPHC can improve survival for PC. Currently, such method of treatment has evolved into a novel approach for PC and might represent the standard care in selected patients (Figure 2)[38-40].

Rationale

Intraoperative use of chemotherapeutic agents to maximize

the efficacy of both surgery and chemotherapy has many advantages. First, surgery can separate the adhesions and remove the bulky tumor, leaving microscopic residual tumors much more susceptible to the killing effect of chemotherapeutic drugs. Second, intraperitoneal chemotherapy has distinct pharmaceutical advantages. In a recent phase I study by Morgan *et al*[41], 36 patients with PC of different origin were treated with intraperitoneal gemcitabine chemotherapy. It was found that the median peak peritoneal concentration was 1116 folds (range, 456-1886) higher than the peak plasma level. This could increase the local drug concentration, thus intensifying its direct antitumor effect while reducing the systemic adverse effects. Such preferential high concentration of drugs in the peritoneal cavity also occurs in many other drugs such as doxorubicin, melphalan, mitomycin C, cisplatin, gemcitabine, mitoxantrone, oxaliplatin, etoposide, irinotecan, paclitaxel, docetaxel, 5-fluorouracil, floxuridine and carboplatin[42]. Third, heat itself has direct detrimental effect on the growth of cancer cells. Cell membrane, cytoskeletons, synthesis of macromolecules, and DNA repair mechanisms are all affected by hyperthermia[38]. Temperature over 43℃ has direct cytostatic effect on human breast cancer cell line MCF-7, ovarian SKOV-3 and hepatocarcinoma HEpG2 cells[43]. Fourth, the synergistic effect of hyperthermia and chemotherapy has also been well-documented. It has been shown that hyperthermia increases the cytotoxicity of some chemotherapeutic agents[43-45]. Because of these advantages, CRS plus IPHC has been increasingly used in the treatment of PC from GC[46], malignant mesothelioma[47], appendiceal cancer[48], CRC and OC[49-60].

Indications, contraindications and patient selection

IPHC is commonly indicated for the treatment of PC with GC, CRC, appendiceal cancer, OC, peritoneal mesothelioma, pseudomyxoma peritonei and malignant ascites, if the patients can stand CRS and IPHC[38]. The contraindications are as follows: (1) patients are medically unfit to undergo the rigors of CRS and IPHC e.g. those with renal and myocardial impairments; (2) there is an extra-abdominal disease; (3) parenchymal hepatic metastases; (4) bulk retroperitoneal disease; (5) the peritoneal tumor is incompletely resected or can not be significantly reduced; and (6) patients aged over 70 years[38]. A clear preoperative staging of PC is necessary for such treatment. Preoperative abdominopelvic CT, MRI, positron emission tomography (PET) or PET/CT, or laparoscopy are adequate procedures[39].

Techniques

Complete CRS is vital in improving the survival of patients with PC, although in some patients with limited PC, partial removal of peritoneum is enough to eliminate macroscopic disease[61]. The goal of complete CRS is to remove all macroscopic peritoneal dissemination. Peritonectomy consists of two big surgical components (parietal and visceral peritonectomy). A midline incision extending from xiphoid process to pubis is sometimes necessary, complete parietal peritonectomy can be achieved by stripping off the parietal peritoneum at the abdominal incision, then

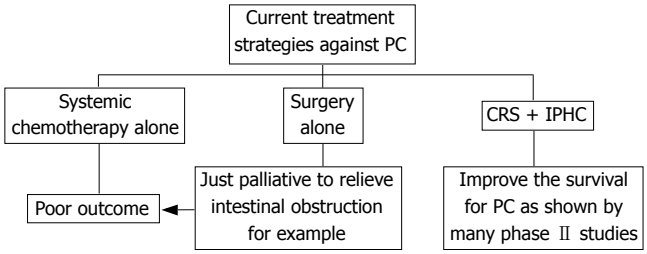


Figure 2 Rationale of CRS plus IPHC in treating PC.

ascending colon, spleen, pancreatic tail, and descending sigmoid colon are mobilized, and all peritoneum lining the lower abdominal wall and pelvis is removed. Visceral peritonectomy includes subtotal colectomy, omentectomy, partial gastrectomy, and resection of mesentery to the extent that they are involved[10].

It is mandatory to examine the greater omentum, undersurface of diaphragm and Douglas pouch as free cancer cells can lodge there due to presence of lymphatic stomata[62]. Micrometastasis on the preserved peritoneum can be managed by intraperitoneal chemotherapy, so complete cytoreduction includes the removal of peritoneum with gross nodules[10,61,62].

After finishing CRS, IPHC is initiated. It has been shown that hyperthermia augments the penetration of anticancer drugs[63], in addition to its synergistic cytotoxicity on cancer cells when combined with some chemotherapeutic drugs such as mitomycin C (MMC) and cisplatin[64]. It is performed either with open or closed techniques, with more advantages of the former due to uniform distribution of the chemotherapeutic agents within peritoneal cavity[10]. In either technique, large volume (5 L-10 L) of saline heated at 42℃-43℃ is introduced into the peritoneal cavity and circulated at a high flow rate of about 10 L/min[10]. The total dose of MMC and CDPP should be 30 mg and 300 mg, respectively with an optimal duration of 60-90 min[10].

ASSESSMENT OF COMPLETENESS OF CRS

There is agreement among many treatment centers that the extent of disease prior to CRS and the completeness of cytoreduction (CCR) score are the principal prognostic indicators for survival[14,38,66-85]. In CCR, the extent of residual disease after CRS is classified into 4 categories: CCR-0 indicating no visible residual tumor; CCR-1, residual tumor $\leqslant$ 2.5 mm in diameter; CCR-2, residual tumor between 2.5 mm and 2.5 cm; and CCR-3, residual tumor > 2.5 cm or confluence of disease present at any site[86]. But those with more diffuse disease (more than 2 quadrants) become worse no matter how good a cytoreduction procedure is. The understanding of a complete cytoreduction may vary according to the disease process, for example, in colorectal PC, complete cytoreduction needs a CCR-0 score[14,66-85] while in pseudomyxoma peritonei, a complete cytoreduction may involve both CCR-0 and CCR-1[39,87].

Table 1 The survival rates of CRS plus IPHC in patients with PC of different malignancies

Authors	Pts *n*	Year	Type of study	Tumor type	Drugs, temperature & duration	Median follow-up time	Survival rate (%) 1 yr	2 yr	3 yr	4 yr	5 yr
Pilati *et al*[90]	34	2003	Phase Ⅱ	Colon ca	MMC 26.2 mg (20.1-31.6 mg) CDDP 193.7 mg (170-241.1 mg), 41.5℃ (41.2℃-42.1℃), 90 min	14.5 mo (6-34 mo)	68	31	-	-	-
Glehen *et al*[81]	56	2003	Phase Ⅱ	CRC, OC, GC, peritoneal Mesothelioma, Pseudomyxoma peritonei, and others	MMC 0.7 mg/kg (max dose 60 mg), CDDP 1 mg/kg (max dose 80 mg), 46℃-48℃, 90 min	544.4 d (133-1680 d)	-	R0: 79.0; R2: 44.7	-	-	-
Verwaal *et al*[67]	54	2003	Phase Ⅲ	CRC	MMC 70 mg, 41℃-42℃, 90 min	21.6 mo	67	44	-	-	-
Witkamp *et al*[60]	29	2001	Phase Ⅰ / Ⅱ	CRC	MMC 35 mg/m^2, 40℃-41℃, 90 min	38 mo (26-52 mo)	82	45	23	-	-
Shen *et al*[84]	77	2004	Phase Ⅱ	CRC	MMC 40 mg, 40.5℃, 120 min	15 m	56	-	25	-	17
Elias *et al*[91]	24	2004	Phase Ⅱ	CRC	LOHP 460 mg/m^2 in 2 L/m^2, 43℃, 30 min	27.4 mo (18.3-49.6 mo)	83	74	65	-	-
Yonemura *et al*[92]	107	2005	-	GC	MMC 30 mg, CDDP 300 mg, etoposide 150 mg, 42℃-43℃	46 mo	-	-	-	-	6.7 all pts; 27 R0 pts
Yonemura *et al*[93]	48	2001	Randomized study	T2-T4 GC	MMC 30 mg, CDDP 300 mg, 42℃-43℃, 60 min	5.5 yr (2.4-10.8 yr)	-	-	-	-	61
Elias *et al*[94]	30	2006	Phase Ⅱ	CRC	LOHP 460 mg/m^2 in 2 L/m^2 of iso-osmotic 5% dextrose, 43℃, 30 min	55 mo (24-80 mo)	97	73	53	49	-
Yan *et al*[75]	30	2006	-	CRC	-	12 mo	72	64	-	-	-
Glehen *et al*[78]	53	2004	Phase Ⅱ	CRC	MMC 40-60 mg, 46℃-48℃, 90 min	60 mo	55	32	-	-	11
Helm *et al*[95]	18	2007	Retrospective review	Recurrent OC	CDDP 100 mg/m^2 or MMC 30-40 mg, 41℃-43℃, 90 min	16.2 mo (6-33 mo)	-	60	-	-	-
Deraco *et al*[96]	33	2004	phase Ⅱ	Pseudomyxoma peritonei	CDDP 25 mg/m^2 per L plus MMC 3.3 mg/m^2 per L, 60 min, 42.5℃	28.6 mo (0.4-72 mo)	-	-	-	-	97
Cavaliere *et al*[97]	69	2003	Phase Ⅱ	CRC	MMC	-	-	-	27	-	-
Glehen *et al*[66]	506	2004	Phase Ⅱ	CRC	MMC/LOHP, 40℃-43℃, 30-90 min	53 mo	72	-	39	-	19
Verwaal *et al*[98]	117	2005	Phase Ⅱ	CRC	MMC 35 mg/m^2, 40℃-41℃, 90 min	46 mo	75	-	28	-	19
Sugarbaker *et al*[72]	70	2006	Phase Ⅱ	CRC	MMC	47 mo	88	-	44	-	32
Helm *et al*[99]	5	2007	Retrospective review	Recurrent endometrial ca	CDDP 100 mg/m^2, 41℃-43℃, 90 min	36 mo	80	80	80	80	-
Zanon *et al*[100]	25	2006	Phase Ⅱ	CRC	MMC 15 mg/m^2, 42℃, 60 min	36 mo	64	40	-	-	-
Piso *et al*[101]	19	2004	-	OC	CDDP 75 mg/m^2, or mitoxantrone 15 mg/m^2, 41.5℃, 90 min	-	-	-	-	-	15
Roviello *et al*[102]	59	2006	-	OC, CRC, GC, Pseudomyxoma, Mesothelioma	MMC 25 mg/m^2, CDDP 100 mg/m^2. (LOHP 460 mg/m^2 in 4 pts with colorectal PC), 41℃-43℃, 60 min	25 ± 21 mo	-	-	-	-	50.8

GC: Gastric cancer; CRC: Colorectal cancer; OC: Ovarian cancer; MMC: Mitomycin C; CDDP: Cisplatin; LOHP: Oxaliplatin.

EFFICACY AND BENEFIT OF CRS PLUS IPHC

It is now clear that CRS plus IPHC improves both the quality of life especially in patients with malignant ascitis[15,103,104], and the survival[15,67]. The results of the most significant studies about the survival rates are summarized in Table 1, which supports the use of this new modality as standard care.

ADVERSE EVENTS OF IPHC

IPHC is associated with a high morbidity rate ranging from 27% to 56%[38]. The adverse events can be classified into CRS-related and chemotherapy-related morbidity[15]. Surgery-related adverse events are abscess, intestinal perforation, fistula, prolonged ileus, bile leakage, pancreatitis, pneumonia, deep vein thrombosis, pulmonary emboli, cardiac insufficiency and cerebral infarcts[11,38].

Multivariate analysis demonstrated that adverse events are related to the stage of PC, the operation duration, the number of anastomoses, and blood loss[38,88].

Main chemotherapy-related side effects are bone marrow suppression and renal insufficiency mostly with use of cisplatin[11]. The mortality rate for IPHC ranges between 0% and 11%, and the most common causes of death are bowel perforation, bone marrow suppression, respiratory failure, methicillin resistant staphylococcus aureus infection, and pulmonary embolism[38].

In spite of the above morbidities, such modality remains the suitable one if not the best at present because the patients with PC might get worse without it as systemic chemotherapy or surgery alone is associated with much poorer outcome. With careful and accurate selection of patients, excluding those who are unfit, it is possible to reduce the morbidities of CRS + IPHC and yield a significantly favorable impact on improving survival of the patients.

FUTURE DIRECTIONS

Recently a panel of 55 experts in PC has reached the consensus, which stated that systemic treatment alone may be no longer appropriate for patients with limited PC from a primary or recurrent colon cancer. CRS plus IPHC as a new treatment strategy have clear advantage in this setting[89]. Such procedure has been approved by the surgical oncology experts on PC from North America, France, Italy, Germany, Holland, Spain and Australia. The consensus also indicated that patients who have isolated PC and are likely to receive a complete cytoreduction, as determined by preoperative CT scans, should undergo CRS; and if complete cytoreduction is achieved, intraperitoneal hyperthermic MMC should be given (at 15-35 mg/m^2, 39℃-42℃, for 60-120 min, either by closed or open instillation), followed by best adjuvant systemic chemotherapy[65].

Several issues have been discussed about the future of this treatment strategy, one of which is how to make such therapy standardized and available to large numbers of patients. The operative procedures required for aggressive cytoreduction are lengthy, challenging and morbid and use a great deal of hospital, blood bank, and operational theater resources[38]. Even though this treatment strategy has been confirmed to be effective in some centers, there is only one randomized clinical trial to definitely establish its efficacy in terms of improving the survival and the quality of life of patients with colorectal PC[39,67]. Currently, a large scale prospective multi-center phase Ⅱ study using CRS and IPHC with MMC followed by modern adjuvant systemic chemotherapy for patients with isolated colorectal PC is in progress. This study involves 66 peritoneal surface malignancy surgeons from 46 institutions in 16 countries. Once completed, this study will provide better evidence as to the efficacy of this treatment modality[89].

It is clear that CRS plus IPHC is not indicated for all patients with PC, and the results achieved by international experts in this field may not be replicated in routine clinical practice. Patients with good performance status, low volume of peritoneal disease, and absence of extra-abdominal metastases are more likely to benefit from the combined treatment. This means early diagnosis, early referral to specialist peritonectomy centers for staging, and prompt intervention are keys to successful management. Only by collaborative efforts from medical oncologists, surgical oncologists, and peritoneal surface malignancy treatment centers can such result be achieved[39].

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In vivo anti-tumor effect of hybrid vaccine of dendritic cells and esophageal carcinoma cells on esophageal carcinoma cell line 109 in mice with severe combined immune deficiency

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Abstract

AIM: To develop a fusion vaccine of esophageal carcinoma cells and dendritic cells (DC) and observe its protective and therapeutic effect against esophageal carcinoma cell line 109 (EC109).

METHODS: The fusion vaccine was produced by fusing traditional polyethyleneglycol (PEG), inducing cytokine, sorting CD34+ magnetic microbead marker and magnetic cell system (MACS). The liver, spleen and lung were pathologically tested after injection of the fusion vaccine. To study the therapeutic and protective effect of the fusion vaccine against tumor EC109, mice were divided immune group and therapeutic group. The immune group was divided into P, E, D and ED subgroups, immunized by phosphate buffered solution (PBS), inactivated EC109, DC and the fusion vaccine respectively, and attacked by EC109 cells. The tumor size, weight, latent period and mouse survival period were recorded and statistically analyzed. The therapeutic group was divided into four subgroups: P, inactivated EC109, D and ED subgroups, which were attacked by EC109 and then treated with PBS, inactivated EC109, DC, and EC109-DC respectively. Pathology and flow cytometry were also used to study the therapeutic effect of the fusion vaccine against EC109 cells.

RESULTS: Flow cytometry showed that the expression of folate receptor (FR), EC109 (C), DCs (D) in human nasopharyngeal carcinoma cell line (HNE1) (B) was 78.21%, 89.50%, and 0.18%, respectively. The fusion cells (C) were highly expressed. No tumor was found in the spleen, lung and liver after injection of the fusion vaccine. Human IgG was tested in peripheral blood lymphocytes (PBL). In the immune group, the latent period was longer in EC109-DC subgroup than in other subgroups, while the tumor size and weight were also smaller than those in ED subgroup. In the therapeutic group, the tumor size and weight were smaller in ED subgroup than in P, inactivated EC109 and DC subgroups.

CONCLUSION: Fusion cells are highly expressed not only in FR but also in CD80. The fusion vaccine has a distinctive protective effect against tumor EC109 and can inhibit the growth of tumor in mice, and its immune protection against tumor attack is more significant.

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Key words: Dendritic cells; Esophageal carcinoma cells; Cell fusion; Immune protection; Immunotherapy

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INTRODUCTION

Dendritic cells (DCs) are the most potential and professional antigen presenting cells, which stimulate the native T cells and play an important role in the T lymphocyte-mediated immunoresponse[1]. It was reported that DCs activated by antigen display a good anti-tumor effect both *in vitro* and *in vivo*[2-4]. We have studied the biological characteristics and induced antitumor immunity

of the cancer vaccine prepared by fusing esophageal carcinoma cell line with DCs derived from the cord blood in a previous study[5]. This study was to develop a fusion vaccine and observe its anti-oesophagus cancer effects on EC109 cells in mice with severe combined immune deficiency (SCID). It is expected that this paper may provide some worthy data for the prevention and treatment of oesophageal carcinoma.

MATERIALS AND METHODS

Tumor cells

EC109 cells were obtained from the Pathological Institute of Shantou University Medical College. Human nasopharyngeal carcinoma cell line (HNE1) was obtained from the Tumor Research Laboratory of Tumor Hospital of Shantou University Medical College.

Isolation of DC

CD34+ hematopoietic stem cells were isolated from the cord blood by Ficoll-Hypaque density-gradient centrifugation using the CD34+ progenitor cell isolation kit and the magnetic cell sorting system (MACS). The CD34+ cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum containing 300 ng/mL human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), 150 μg/mL human tumor neucrosis factor α (rhTNF-α) and 200 ng/mL human stem cell factor (rhSCF) at 37℃, in an atmosphere containing 5% CO_2 for two weeks and then a large number of DCs were harvested.

Cell fusion

DCs were mixed with EC109 at a 5:1 ratio and incubated in serum free RPMI1640 containing 50% polyethylene glycol and the fusion cells were labeled with HLA-DR MicroBeads for 35 min at 6℃-12℃. The cells were centrifuged at 1000 r/min for 10 min. By using a MiniMACS separation column to enrich the magnetically positive cells, the enriched HLA-DR+ cells were washed, seeded in 12-well plates and cultured overnight. The adherent cells were regarded as fused cells EC109-DC.

Fusion cell verification

Peripheral blood lymphocytes (PBL) were isolated from the peripheral blood of normal donors by Ficoll-Hypaque density-gradient centrifugation. PBL, DC, EC109 and HNE1 were planted in RPMI 1640 medium supplemented with 10% fetal calf serum, washed with PBS and incubated with FA-FITC (which was obtained from Peixuan G, Purdue University, USA) for one hour. PBL were set up for negative cells and HNE1 for masculine cells as previously described[6]. EC109, DCs and EC109-DC were washed with PBS and incubated with FA-FITC and CD80-PE. Fluorescence intensity and positive cell percentage were analyzed by fluorescence-activated cell sorter analysis using FACScan (Beckman Coulter).

Experiment in SCID mice

Preparation of SCID mice: SCID mice, 6-8 wk old, were purchased from the Animal Research Center of Zhongshan University. The animals were kept in micro-isolator cages in a separate barrier facility under a well-controlled pathogen-free environment with ambient temperature monitored and cycles of light and darkness regulated. The mice were fed with laboratory chow and water at the Tumor Research Laboratory of Tumor Hospital of Shantou University Medical College. The guidelines for animal care and use were approved by the experiment animal monitor of Guangdong Province.

Oncogenicity of fusion vaccine: Experiments were divided into sets Ⅰ and Ⅱ. There were 5 mice in set Ⅰ. Live fusion vaccine (0.2 mL) was then injected into vena caudalis of the 5 mice. Twenty-five mice in set Ⅱ were divided into 5 groups (A-E). Group A was injected intraperitoneally with 1×10^6 cells each of EC109-DC. The other 4 groups were injected with the same quantity of cells of EC109 + DC, EC109, DC and PBS. Whether tumor was induced in the mice of set Ⅰ was checked. The development time and size of the tumor, as well as the survival time of the mice were observed every day for 60 d. Organs, such as the spleen, liver and kidney were removed and fixed in neutral formalin, embedded in paraffin and stained with hematoxylin and eosin (HE) for routine light microscopic study. The mice in set Ⅱ were killed on d 28 after inoculation. The removed tumors were weighed and fixed in neutral formalin, embedded in paraffin and stained with HE for routine light microscopic study.

Reconstitution of SCID mice: Human lymphocytes to be transferred in SCID mice were isolated from the whole blood obtained from healthy adult volunteers as previously described[7-11]. Human peripheral blood lymphocytes (PBL) were isolated by Ficoll-Paque gradient centrifugation under sterile conditions. PBL were planted in RPMI 1640 medium supplemented with 10% fetal calf serum. The culture flasks were incubated in a humidified atmosphere containing 5% CO_2 at 37℃. Thirty SCID mice were divided into two groups. Twenty-five mice in PBL group were injected intraperitoneally with 5×10^7 PBL. The other 5 mice in PBS group were injected with the same volume of PBS as a control group. Blood was obtained from the vena caudalis of mice on d 0, 7, 14, 21 and 28, and then centrifuged. The sera were collected for detecting the level of human IgG by avidin-biotin (ABG) ELISA. The successful marking of reconstitution is that the human lymphocytes or human IgG could be found in the peripheral blood of SCID mice.

Induction of anti-tumor immunoprotective effect: Twenty SCID mice with successful immune system reconstruction were divided into P, E, D and ED subgroups. PBS (0.2 mL) was injected into the vena caudalis of mice. EC109, DC and the fusion vaccine were inactivated with 1×10^6 cells each time, twice a day for 3 d. Five days later, all the mice were injected into the right armpit with 5×10^6 of live EC109. The tumor size, weight, latent period and survival period of mice were recorded at the same time point every day for 50 d. Two mice in each group were sacrificed 28 d after tumor attack. Tissues of the spleen, liver and kidney, were fixed in neutral formalin,

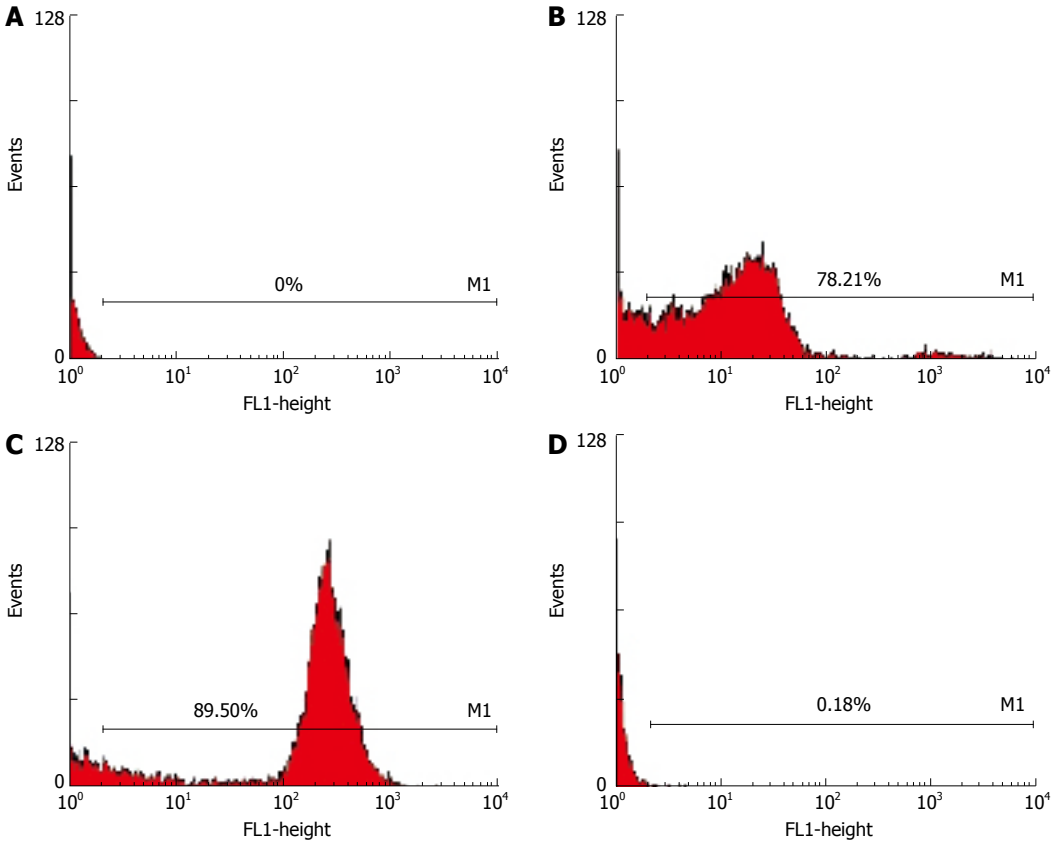


Figure 1 Expression of folate receptor on EC109 and DCs. PBL (**A**) was set up for negative cell and HNE1 for masculine cell. Analyses by flow cytometry, HNE1 (**B**) with expression of FR was 78.21%, EC109 (**C**) was 89.50%, and DCs (**D**) was 0.18%.

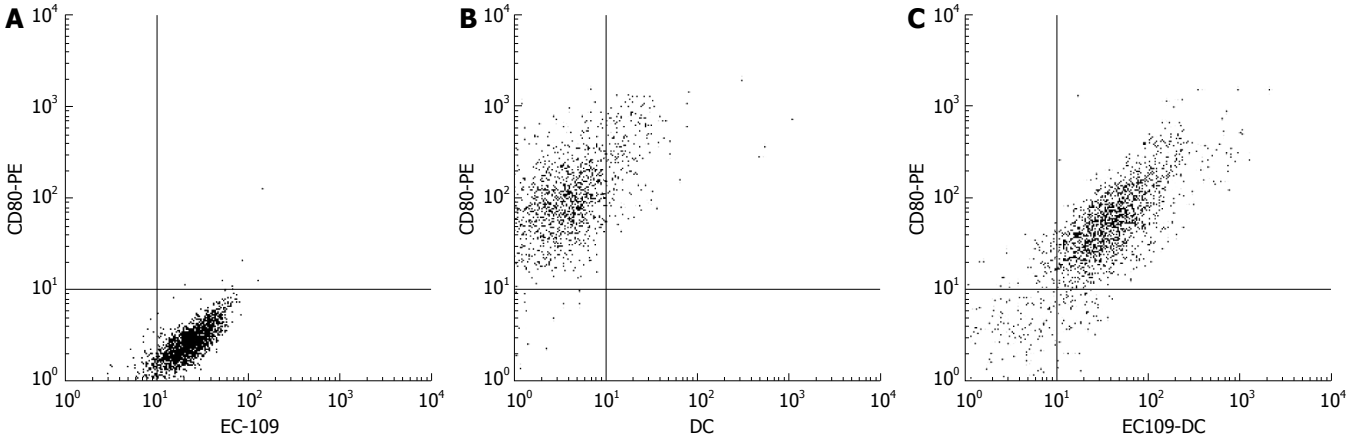


Figure 2 Expression of FR (**A**), CD80 (**B**) and EC109-DC (**C**).

embedded in paraffin and stained with HE for routine light microscopic study.

Anti-tumor therapeutic effect: The EC109 cells at the logarithmic growth phase were collected and washed with PBS. The cell density was adjusted to 5×10^6/mL. Twenty SCID mice with successful immune system reconstruction were injected into the right armpit with 1×10^6 cells of live EC109. The model was successful when the tumor grew as big as a grain. The 20 mice in the model were divided into P, E, D and ED subgroups. PBS (0.2 mL) was injected into the vena caudalis. EC109, DC and the fusion vaccine were inactivated with 1×10^6 cells each time, twice a day for 3 d. Tumor size, weight, latent period and survival period of mice were recorded at the same time point every day. One mouse in each group was sacrificed 28 d after immunity acquirement. Others were observed for an additional 50 d. Organs, such as the spleen, liver and kidney, were fixed in neutral formalin, embedded in paraffin and stained with HE for routine light microscopic study.

Statistical analysis

SPSS10.0 was used for data variation analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of EC109-DC fusion cells

After fusion of EC109 and DCs, the resulting heterokaryons showed adherent growth and irregular shape. Flow cytometry displayed that the expression of FR, EC109 and DCs was 78.21%, 89.50% and 0.18%, respectively in HNE1.

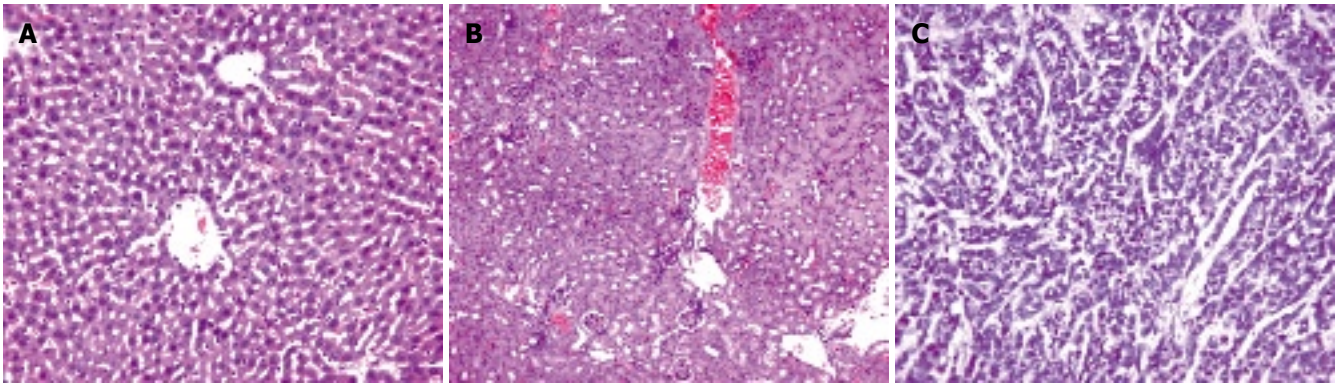


Figure 3 No formation of tumor in the liver (**A**), in the kidney 60 d after injection of EC109-DC into the vena caudalis (**B**) (HE, 20 × 10) and in tumor tissue (**C**) of SCID mice 28 d after injection of EC109 into the abdominal cavity (HE, 10 × 10).

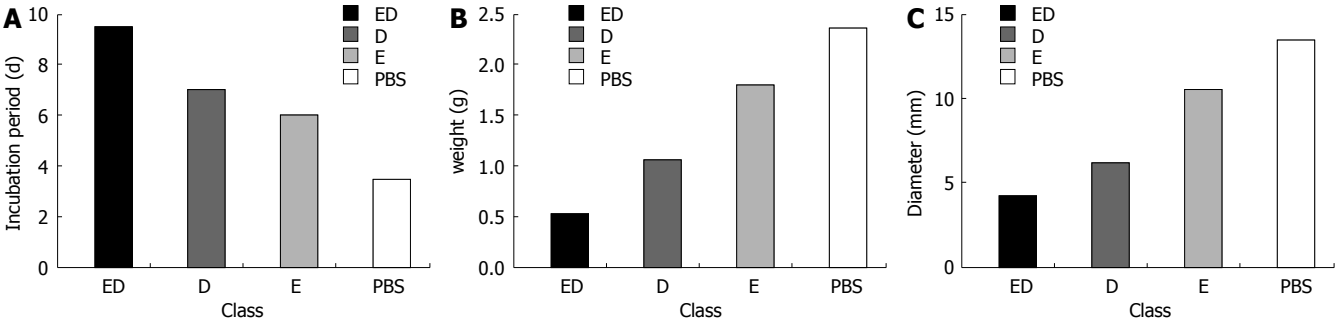


Figure 4 Incubation time (**A**), weight (**B**) and diameter (**C**) of tumors after attacked by EC109.

The fusion cells were highly expressed not only in FR but also in CD80 (Figures 1 and 2).

Oncogenicity

There was no formation of tumor 60 d after injection of EC109-DC in group 1. No formation of tumor tubercles was discovered in the heart, liver, lung, kidney and spleen (Figure 3).

There were no ascites and lump organization 28 d after injection of EC109-DC in group 2. However, hemorrhagic ascites was discovered, grey tumor tissues were generated generally and widely adhered to the ambient organs in groups of EC109 + DC and EC109. The tumor tissues were mostly distributed on the abdominal wall, diaphragmatic muscle, liver and pelvic cavity with a diameter of 1-20 mm. Under light microscope, the size and shape of cancer cells were not extremely consistent, but polygonal and karyolobism lost the characteristics of epithelial cells in normal esophagus (Figure 3).

Examination of reconstitution

During the experiment, human IgG was tested in all the PBL groups and its highest level was 2580 μg/mL, compared with the PBS group ($P < 0.05$, Table 1).

Anti-tumor immunoprotective effect

The incubation period of tumor cells after attacked by EC109 was P < E < D < ED (Figure 4A). The tumor weight and size were ED < D < E < P (Figure 4B and C). Except for 2 mice which were killed on d 28, the other

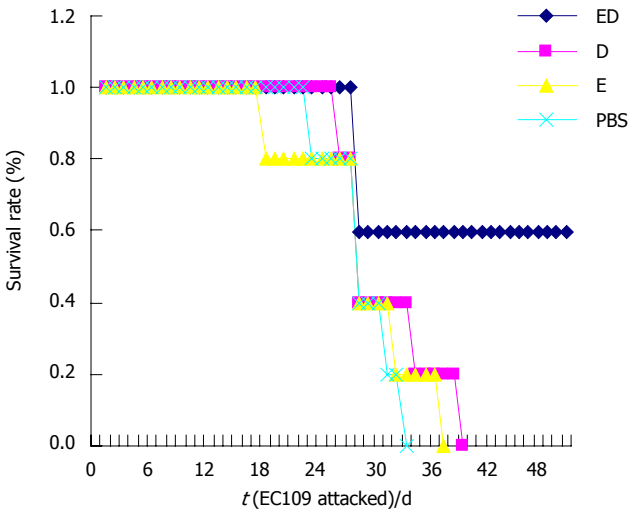


Figure 5 Death and life span in immune, ED and PBS groups.

mice in the ED group survived and their life span was obviously longer ($P < 0.05$). Compared with the PBS group, death occurred in the treatment group and the difference in the life span between the two groups was not significant ($P > 0.05$, Figure 5).

Pathology: Circlar, slippery and pellicular tumors in ED group were distended and movable with neither wide conglutination nor metastasis. Grey and hard tumors with an uneven surface were found in other groups. Some

Table 1 Human IgG level in SCID mice (μg/mL)

Class	*n*	D0	D7	D14	D21	D28
PBS	5	0.12 ± 0.03	0.05 ± 0.03	0.06 ± 0.01	0.09 ± 0.04	0.16 ± 0.12
PBL	25	0.21 ± 0.10	59.28 ± 12.61	260.82 ± 36.16	353.40 ± 69.23	482.7 ± 185.15

$P < 0.05$ *vs* PBS group.

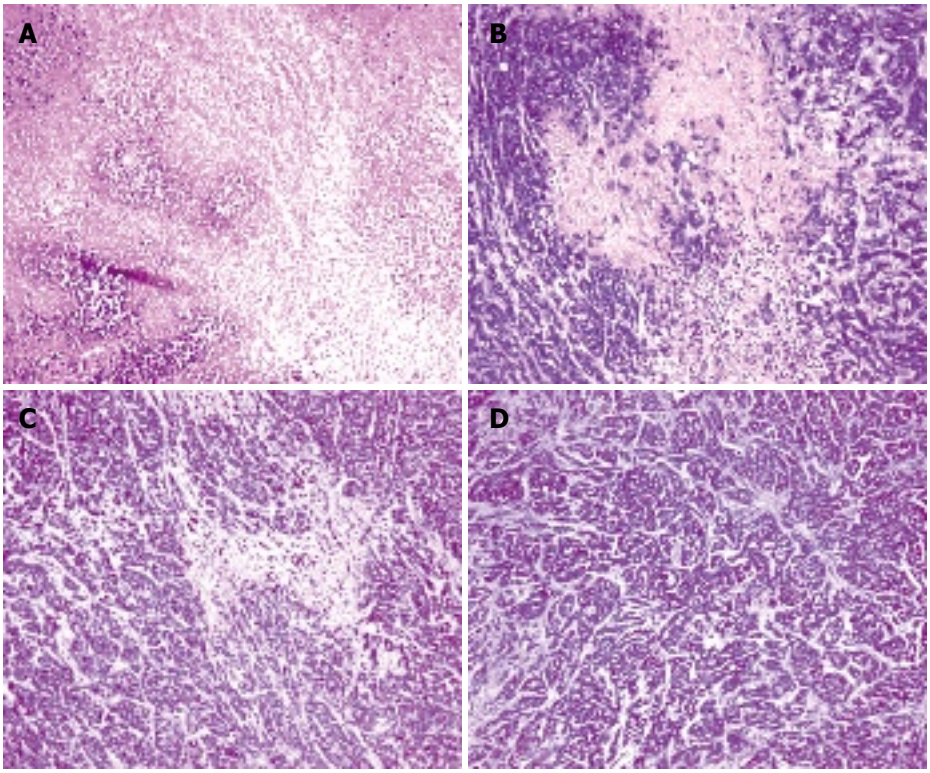


Figure 6 Tumor tissue 28 d after attacked by EC109 showing big necrosis in ED group (**A**), lamella necrosis in D-group (**B**), mottling necrosis in E-group (**C**) and unobvious necrosis in P-group (**D**) (HE, 10 × 10).

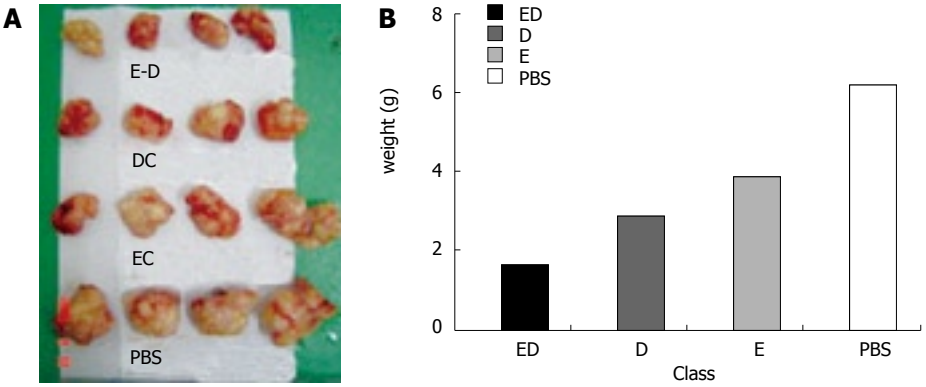


Figure 7 Tumor size (**A**) and weight (**B**) in therapeutic group after treatment of EC109-DC, DC, inactivated EC109 and PBS.

tumors were adhered to the ambient structure, even with distant metastasis. Tumor tissue sections were grey in color with diffuse punctiform necrosis. Under light microscope, tumor tissues from the ED, D and E groups attacked by EC109 for 28 d, except for P group, had different necrosis changes (Figure 6).

Immunotherapy: After treatment of EC109-DC, DC, inactivated EC109 and PBS, the tumor size was DE < D < E < P. On d 50, the growth time of tumors had no remarkable difference but the tumor weight did ($P < 0.05$, Figure 7).

The texture of tumors with a smooth surface was harder, partially adhered to the chest wall and the tumor tissue section was grey in color with diffuse punctiform necrosis. Under light microscope, the tumor tissue had different necrosis changes (Figure 8).

DISCUSSION

In this experiment, the fusion vaccine EC109-DC, was made from amalgamation of DC, which was induced by combining cytokine from CD34+ hematopoietic stem cells isolated from the cord blood, and the EC109 by traditional

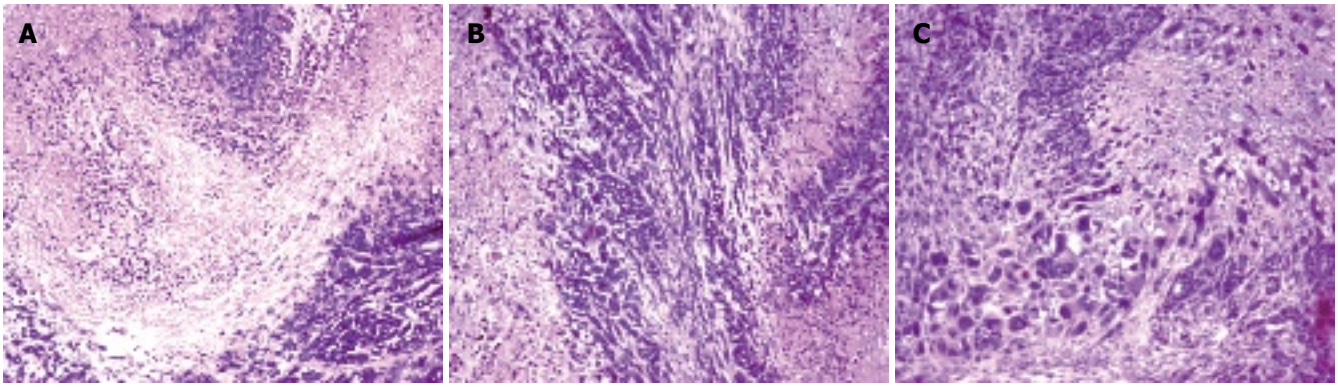


Figure 8 Tumor tissue 28 d after attacked by EC109 showing slight necrosis (**A**), lamella necrosis (**B**) and mottling necrosis (**C**) in therapeutic group (HE, 10 × 10) .

PEG fusing. The fusion vaccine, EC109-DC, integrated the protein, polypeptide and nucleic acid of EC109 and even the whole live cells with the antigen presenting DC, thus it can give immunoreaction to polyvalency tumor antigen, showing all possible antigens to EC109. With no specific antigen to the esophageal carcinoma cell line, it can effectively induce anti-tumor immune response. Consequently, it is more effective and practical to fuse vaccine with DC than a single antigen in theory[12-14]. Some curative effects of fusion vaccine with DC have been seen in clinic experiments[15-25]. However, it cannot be brought into full use due to its lower fusion efficiency, shortage of effective methods of separation and purification as well as the difficulty in obtaining an enough-quantity and high-quality cancer vaccine by fusing cancer cell line with DC.

In this study, we developed the tumor fusion vaccine with HLA-DR mark and Mini MACS sorting method. The tumor fusion vaccine, EC109-DC, was double marked with FA-FITC and CD80-PE. The vaccine's phenotype was accredited by flow cytometry amb-masc mark. After detection, the vaccine highly expressed FR and CD80 simultaneously. The principle of HLA-DR+ cells separated by Mini MACS separation unit is to abrupt the HLA-DR+ cells and the HLA-DR- cells which mark the magnetic by coupling the antibody to the cells, and HLA-DR monoclonal antibody to the HLA-DR+ cell membrane's SA idio-discrimination. The results of our study have not only proved the successful fusion of DC with EC109, but also the separation effect of MACS. This may provide an effective way for the effective purification and separation of the vaccine, as well as for gaining an enough quantity and high-quality of hybrid DC.

Although the fusion vaccine was inoculated in the right abdominal cavity of SCID mice, we could not see hemorrhagic ascites and tumor tubercules in the abdominal cavity. There were hemorrhagic ascites and adhesive tumor organism in the control group. The fusion vaccine was injected into the SCID mice through vena caudalis, no tumor nodus was found in the liver, kidney and spleen of mice, indicating that the fusion vaccine has lost the oncogenicity of parent tumor cells, and is safe and reliable for its application *in vivo*.

In anti-tumor protective immunity *in vivo* experiment, tumor formulation was delayed in the ED and D groups compared with the immunoprotection groups. The time of tumor formulation in P group was the shortest. The tumor was the smallest and lightest in ED group, and the biggest and heaviest in D group, indicating that the fusion vaccine has the best immunity protective function. Our study used the cell-fusion technique to increase the number of cells fused with DC, which highly express MHC, co-stimulate molecules and adhesion molecules and also produce IL-12 and tumor cells which are short of information molecules, form the EC109-DC which change the bionomics of tumor cells. The fusion vaccine was re-infused *in vivo* in which DC carry all esophagus cancer cell antigen information. The fusion cells retain the MHC and co-stimulatory molecules in DC. The EC109-DC activate the CD4+ T and CD8+ T cells as MHC- I and MHC-Ⅱ, secrete homologous cytokines and activate the anti-tumor immune response, thus inducing special anti-tumor immunity reaction more effectively.

Sixty days after treatment of tumor-bearing mice with the fusion vaccine, EC109, the size of tumor in ED group became obviously smaller than that in the control group. The tumor surface in ED group was smooth and divided by circum-tissues and had no conglutination. However, the tumor surface in control group was coarse and adhered to the chest wall and muscle tissues had no clear demarcation. Macro-lamellar necrosis was observed in ED group under light microscope, while punctiform necrosis was found in PBS group, indicating that the fusion vaccine has certain inhibitory effect on the tumor growth, which is in agreement with previous reports[26,27]. At the same time, active immunotherapy for the tumor-bearing mice reduced the growth of the lump body, and the sphacelus became obvious in tumor *in vivo*, demonstrating that such immunity treatment has its certainly value and even cures the tumor-bearing mice if it is used in combination with operation or chemotherapy.

Tumors can escape the host immunity surveillance because tumor cells cannot express the information molecule required by the host immunity system. It was reported that induction of CD8+, CD4+ and B cells is also one of the most effective method to prevent and control chronic infections, diseases, tumors[28]. The immunoreactions in the host are to capture its antigen by APC. After processing, the antigen information is present in T and B lymphocytes, stimulating a series of specific cellular immunologic response and humoral

immunoresponse. It is, therefore, directly related with the induction of immunological activation or immunological tolerance, and the key to the process is APC. DCs are a kind of the strongest APCs in which antigen processes and functions[29,30], and can stimulate the native T cells. DCs are in a unique state of immune response.

This experiment adopted the technology of cell fusion to make DCs, which highly express the major histocompability complex (MHC), co-stimulate adhesion molecule, and produce IL-12, identify cells fused with tumor cells which are short of the information molecule. All these not only change the biological characteristics of tumor cells, but can effectively process presentation antigen and activate the anti-tumor immune response, and the host obtains the immunity protection strength by the resistance to parent tumor attack. During the procedure, it directly fuses the esophageal carcinoma cells (EC109) and DCs, passes the link of DCs insufficient to recognize EC109 which are low in immune function, causes the direct processing and presentation of tumor antigen in DCs.

At the same time, the fusion vaccine excretes corresponding cell factors, possibly combines the cell molecule with the specific acceptor on DCs surface, and increases the stimulation to DC, activates the low function of DCs, and also its function on antigen presentation. On the other hand, the conjugated tumor antigen is stably expressed on the cell surface, which also may be used as the extraneous antigen to the major histocompatibility complex (MHC-Ⅱ), then activates the CD4+ Th, and induces the production of different antibodies. According to the difference in its secretion of Th1 and Th2 cell factors, the activated CD4+ Th can adjust the balance of immunity, enhance the intensity of cellular immunologic response and (or) humoral immunoresponse response. The tumor antigen to EC109 itself just lies in the DCs, which can be effectively captured by DCs, then presented to MHC- Ⅰ, strengthens the reaction of CD8+ CTL, CD4+ Th cells, and the intensity of the cellular immunologic response and (or) humoral immunoresponse response. The fusion vaccine not only remarkably stimulates initial (naive) multiplication of T cells, but also confers upon the activated T cells for certain tumor memory, which may massively activate and expand under the situation of more contacting antigens[31,32].

In addition, the fusion vaccine also participates in the nonspecific immunity. DCs which fuse tumor cells excrete cytokines, such as IL-1, IL-6, IL-8, IL-12, TNF-α, IFN-α and GM-CSF. Among them, IL-6 dissolves tumor cells by activating the complement mannose-binding lectin(MBL) pathway. The other cytokines such as IFN-α, can directly activate NK cells. After activation under the effect of chemotactic factors, the NK cells migrate to the tumor focus and directly kill and wound the responsive tumor cells, with no need to stimulate antigen and other factors. Inactivated macrophages have no lethal effect on tumor cells and can be activated by the stimulus of inflammation or other factors. LK secretes T cells and IFN. The activated macrophages as effector cells have no specificity to kill and inhibit tumors, but may have effect factors killing target cells, including hyperoxide, nitrogen monoxide, TNF and LYS, *etc*. Through the ADCC way, macrophages can also kill and wound target cells. In this experiment, the anti-tumor function of the fusion vaccine is due to the co-effect between specific immunity and non-specific immunity.

In conclusion, the fusion vaccine simultaneously expresses EC109 and DC specific antigen. Meanwhile, the fusion vaccine of DCs and EC109 has no *in vivo* oncogenicity, and has lost its parent characteristics of EC109 cells with malignant growth, becoming safe and reliable. Furthermore, the fusion vaccine has resistant functions against the attack of EC109 cells. Moreover, the fusion vaccine can restrain tumor growth in mice. The fusion vaccine may become a useful tool in preventing malignant tumors.

COMMENTS

Background

The present study demonstrated that immunization strategies for cancer vaccines based on mature dendritic cells (DCs) could present tumor peptide and induce specific anti-cancer immune response. No report is available on the fusion vaccine for human esophageal carcinoma cells fused with DCs derived from the cord blood.

Research frontiers

We have studied the biological characteristics of cancer vaccine and induced its anti-tumor immunity.

Innovations and breakthroughs

The fusion vaccine of esophageal carcinoma cells and DCs was developed. Its protective and therapeutic effect on EC109 cells was studied. The EC109 highly expressed FR and DCs hardly express the FR.

Applications

To summarize the actual application values, the tumor vaccine could become a useful tool for preventing malignant tumors.

Peer review

This manuscript is an original research article on the protective effect of the fusion vaccine. The findings are of great interest and the data are convincing.

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Localization and translocation of RhoA protein in the human gastric cancer cell line SGC-7901

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Abstract

AIM: To elucidate the localization of RhoA in gastric SGC-7901 cancer cells and its translocation by lysophosphatidic acid (LPA) and/or 8-chlorophenylthio-cAMP (CPT-cAMP).

METHODS: Immunofluorescence microscopy was used to determine the localization of RhoA. Western blotting was used to detect both endogenous and exogenous RhoA in different cellular compartments (membrane, cytosol, nucleus) and the translocation of RhoA following treatment with LPA, CPT-cAMP, or CPT-cAMP + LPA.

RESULTS: Immunofluorescence staining revealed endogenous RhoA to be localized in the membrane, the cytosol, and the nucleus, and its precise localization within the nucleus to be the nucleolus. Western blotting identified both endogenous and exogenous RhoA within different cellular compartments (membrane, cytosol, nucleus, nucleolus). After stimulation with LPA, the amount of RhoA within membrane and nuclear extracts increased, while it decreased in the cytosol fractions. After treatment with CPT-cAMP the amount of RhoA within the membrane and the nuclear extracts decreased, while it increased within the cytosol fraction. Treatment with a combination of both substances led to a decrease in RhoA in the membrane and the nucleus but to an increase in the cytosol.

CONCLUSION: In SGC-7901 cells RhoA was found to be localized within the membrane, the cytosol, and the nucleus. Within the nucleus its precise localization could be demonstrated to be the nucleolus. Stimulation with LPA caused a translocation of RhoA from the cytosol towards the membrane and the nucleus; treatment with CPT-cAMP caused the opposite effect. Furthermore, pre-treatment with CPT-cAMP was found to block the effect of LPA.

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Key words: RhoA; Localization; Translocation; Nucleolus; Gastric cancer cell

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INTRODUCTION

RhoA, with molecular mass of 21 kDa, is the most extensively studied member of the Rho GTPase family belonging to the Ras superfamily of small G proteins. The Rho GTPase family consists of at least 11 members sharing more than 50% identity, the best known of them being Cdc42, Rac1, and RhoA. RhoA has been reported to regulate many biological activities including the formation of stress fibers and focal adhesions[1].

Like other small G proteins, RhoA is inactive in its GDP-bound and active in its GTP-bound form, these two forms can be converted by GDP/GTP exchange or GTPase reactions[2-5]. The former and latter reactions are regulated by GDP/GTP exchange protein and GTPase activating protein, respectively. Two types of GDP/GTP exchange proteins are known: one is a stimulatory type named GDS, and the other is an inhibitory type named GDI. GDS stimulates the dissociation of GDP from and the subsequent binding of GTP to each substrate small G protein, whereas GDI inhibits both reactions[6-15]. In addition to the action of GDS and GDI regulating the GDP/GTP exchange reaction, both GDP/GTP exchange proteins regulate the translocation of each substrate small G protein between the cytosol and membranes.

Lysophosphatidic acid (LPA), a commonly used RhoA activator, is a serum-born phospholipid with hormone and growth factor-like properties, which is formed by and released from activated platelets and which can be

generated by the action of secretory phospholipase A2[16,17]. Previous studies showed that it is able to induce the translocation of RhoA from the cytosol to the membrane accompanied with the activation of RhoA[18], probably *via* conversion of the GDP-bound into the GTP-bound form.

cAMP is a well-known classical secondary messenger, and a cAMP-dependent protein kinase A (PKA) phosphorylates RhoA in its C-terminal region on serine residue 188[19]. The cAMP signal mediates down-regulation of RhoA activity[20]. It was reported that cAMP could induce the translocation of RhoA from the membrane to the cytosol accompanied with the inactivation of RhoA[19]. Moreover, translocation of RhoA was shown to be caused by stabilizing the RhoA/Rho GDIs complex by phosphorylating GTP-bound RhoA[21]. Phosphorylation enhances translocation of RhoA to the cytosol through increased interaction of GTP-bound RhoA with Rho-GDI, possibly terminating its activity.

We here describe RhoA to be localized in the nucleus of SGC-7901 cells and a cross talk between LPA/RhoA-mediated and cAMP/PKA mediated signal transduction pathways[22]. Whether there is interaction between LPA induced and cAMP induced RhoA distributions in different cellular compartments deserves further investigation. Our experiments were designed to explore the localization of RhoA and to investigate the effects of a treatment with LPA, cAMP, and cAMP + LPA.

MATERIALS AND METHODS

Cell lines

The human gastric cancer cell line SGC-7901 and human cervix cancer cell line HeLa were provided by the Institute of Cell Biology (Shanghai, China).

Reagents

Dulbecco's Modified Eagle Media (DMEM) was purchased from GIBCO (Grand Island, NY), new-born calf serum (NBCS) from Minhai Bio-engineering C (Lanzhou, China), the antibody against RhoA from Santa Cruz Biotechnology (Santa Cruz, CA), the antibody against GAPDH (glyceraldehyde phosphate dehydrogenase) from Kangcheng (China), the horseradish peroxidase (HRP)-conjugated secondary antibody from Jackson ImmunoResearch Laboratories (West Grove, PA), the cell transfection reagent Lipofectamin 2000, TRIzol reagent, and reverse transcription kit from Invitrogen (Carlsbad, CA), the cellular permeable cAMP analog 8-chlorophenylthio-cAMP (CPT-cAMP) from Calbiochem, LPA and nuclear fluorochrome Hoechst 33342 from Sigma (St. Louis, Missouri), electrochemiluminescence (ECL) reagents from Amersham Biosciences (Buckinghamshire, England), and pGEmol/L vector from Promega (USA). Plasmid DNA constructs of RhoA pcDNAEE-wt (wild type of RhoA), pcDNAEE-63L (a constitutive active form of RhoA), and pcDNAEE-N19 (an inactive mutant of RhoA) were kind gifts from Dr. Renate Pilz, University of California, San Diego.

Cell culture and transfection

SGC-7901 cells were cultured in DMEM supplied with 10% NBCS. The medium was changed every two days and the cells were subcultured at confluence. For transfection, the cells were subcultured the day before the process. The seeding amount of cells was adjusted to attain a density of 80%-90% confluence on the day of transfection. Transfection was performed according to the instruction of the manufacturer.

Immunofluorescence microscopy

The cells grown on cover slips were incubated to reveal nuclei with 0.2 μmol/L Hoechst 33342 for 10 min before fixed with freshly prepared 40 g/L paraformaldehyde in PBS at 4℃ overnight (o/n). After being penetrated with 30 mL/L Triton X-100 and blocked with 30 g/L BSA, the cells were incubated with primary antibodies at 4℃ o/n, and then with FITC- or TRITC-conjugated second antibodies for 1 h at room temperature (RT), with three washes after each incubation. The morphologic changes of the cells were analyzed by fluorescence microscopy.

Membrane, nucleus, and cytosol preparation

According to the method from Jeffrey C. Chen *et al*[23], four 100 mm plates of cells were prepared, one of them considered as control, and the others treated with LPA (1 μmol/L, 15 min), CPT-cAMP (250 μmol/L, 30 min), or CPT-cAMP (250 μmol/L, 30 min) followed by an incubation with LPA (1 μmol/L, 15 min). Cells were extracted by Dounce homogenization in 10 mmol/L Hepes pH 7.5, 2 mmol/L EDTA, 1 mmol/L $MgCl_2$ (HEM buffer). The homogenate was centrifuged at 500 *g* at 4℃ for 5 min to obtain nuclear proteins, and the supernatant was centrifuged at 37000 *g* at 4℃ for 30 min. The supernatant and pellet from the second centrifugation are referred as cytosol and membranes, respectively, with the membrane preparation washed twice in HEM buffer to remove contaminating cytosol. Protein concentrations were determined and equal amounts of protein from each preparation (30 μg) were subjected to SDS-PAGE/Western blotting using mouse monoclonal anti-RhoA (1:200) and GAPDH (1:5000) antibodies.

Purification of nucleoli from SGC-7901

Nucleoli were purified using a procedure adapted from previously published protocols[24]. For a typical experiment, 1×10^8 SGC-7901 cells were plated onto 245 mm Petri dishes in DMEM containing 10% NBCS. Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37℃. At 80% confluence, cells were washed with cold PBS, and scraped off on ice. Cells were collected by centrifugation at 500 *g* at 4℃ for 5 min. Cells were resuspended in 5 mL hypotonic buffer (10 mmol/L Tris-HCl pH 7.4, 10 mmol/L NaCl, and 1 mmol/L $MgCl_2$) and incubated on ice for 30 min. Cell lysis was performed by addition of a final concentration of 0.3% Nonidet P-40 (Roche Applied Science, Mannheim, Germany) and homogenization was performed using a 0.4 mm clearance Dounce homogenizer (Kimblr/Kontes, Owens, IL) for 15 times on ice. Nuclei were collected by centrifugation at 228 *g* at 4℃ for 5 min and resuspended in 3 mL 0.25 mol/L sucrose-containing 10 mmol/L $MgCl_2$. Nuclei were centrifuged at 1430 *g* at 4℃

for 5 min through a 0.35 mol/L sucrose cushion containing 0.5 mmol/L $MgCl_2$ and resuspended in 3 mL 0.35 mol/L sucrose-containing 0.5 mmol/L $MgCl_2$ and then sonicated on ice for ten bursts of 10 s with 1 min intervals between them. Nuclei were then purified by centrifugation at 2800 *g* at 4℃ for 10 min through a 0.88 mol/L sucrose cushion containing 0.5 mmol/L $MgCl_2$. Nuclei were washed with 500 μL 0.35 mol/L sucrose containing 0.05 mmol/L $MgCl_2$ twice, and centrifuged at 2800 *g* at 4℃ for 10 min. Purified nucleoli were resuspended in 50 μL 0.35 mol/L sucrose containing 0.05 mmol/L $MgCl_2$ for further analyses.

Protein extraction from Hela cells in interphase and motosis

Hela cells were seeded on four 100 mm plates and treated with 5 μg/mL Taxol for 12 h. Cells in mitosis phase were collected by "shake off", and the cells left on plates considered in interphase were scraped. Cells were extracted with 500 μL RIPA (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L PMSF, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), incubated on ice for 30 min, and then centrifuged at 14000 *g* at 4℃ for 15 min to obtain total protein extracts. Protein concentrations were determined and equal amounts of protein from each preparation (30 μg) were subjected to SDS-PAGE/Western blotting using mouse monoclonal anti-RhoA (1:200) and GAPDH (1:5000) antibodies.

Western blotting

SDS-PAGE gels of different concentrations were cast according to the molecular size of target proteins. Sample proteins were run on 10% SDS-polyacrylamide gels, and were blotted onto polyvinyl difluoride (PVDF) membrane. The PVDF membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in TBS-T for 1 h at room temperature (RT). Membranes were incubated with the primary antibody at 4℃ o/n, and with the secondary antibody for 1 h at RT, with three washes after each incubation. Electrochemiluminescence reagents were used to show the positive bands on the membrane. Briefly, same volumes of solution A and solution B were mixed and added to the protein side of the membrane. The incubation was 5 min at RT. The exposure time of the first film was 15 s. The exposure time of the second film was adjusted according to the intensity of the signal on the first film. The bands on film were analyzed with GeneSnap/ Gene Tool software from Syngene (Cambridge, UK).

RNA isolation, reverse transcription, and real-time quantitative PCR

To detect the difference in expression of RhoA gene between interphase and mitosis phase in HeLa cells, total RNA of interphase and mitosis cells was isolated using TRIzol reagent. cDNA was synthesized in a 20 μL reaction containing 4 μg of total RNA and SuperScript™Ⅱ RT according to the manufacturer's instructions. Primers designed for amplification of the human RhoA gene and GAPDH gene were forward 5'-GGCTGCCATCCGGAA GAAA-3' and reverse 5'-CACAAGACAAGGCACCC AGA-3' and forward 5'-GGATTTGGTCGTATTGG G-3', and reverse 5'-GGAAGATGGTGATGGGAT T-3', respectively. The 25 μL reaction contained 0.5 μL cDNA, 10 pmol/L of each primer, 2 mmol/L $MgCl_2$, 2.5 μL of 200 nmol/L dNTP, and 1 unit of Taq DNA polymerase. The thermal cycle profile for PCR was 94℃ for 5 min followed by 35 cycles of 30 s at 94℃, 30 s at annealing temperature (63.8℃ forRhoA and 55℃ for GAPDH), and 30 s at 72℃ with a final 10 min incubation at 72℃. The amplified fragments of the target genes were cloned into pGEmol/L vector and DNA sequencing was performed by Gene Co. Ltd. (Shanghai, China).

For real-time quantitative PCR, standard curves were prepared by serial dilution of pGEM-RhoA and pGEM-GAPDH vector. Reactions of 25 μL included 1 μL of 1:1500 SYBR Green Ⅰ, 0.5 μL cDNA, 10 pmol of each primer, 2 mmol/L $MgCl_2$, 2.5 μL 200 nmol/L dNTP, and 1 unit of Taq DNA polymerase. To compensate for variations of input RNA amounts and efficiency of reverse transcription, an endogenous 'housekeeping' gene (GAPDH) was also quantified and used to normalize the results. The thermal cycle profile for PCR was 94℃ for 2 min followed by 35 cycles of 30 s at 94℃, 30 s at annealing temperature (63.8℃ for RhoA and 55℃ for GAPDH) and 30 s at 72℃. After the cycling of PCR assay was completed, the samples were subjected to a temperature ramp (from 45℃-95℃ at 0.5℃/2 s) with continuous fluorescence monitoring for melting curve analysis. Analyses were done in triplicates and all reactions were repeated six times independently to ensure the reproducibility of the results. Data were analyzed by Rotor-Gene real-time analysis software (Rotor-Gene 2000, CR, Australia). For each sample the amplification plot and the corresponding dissociation curves were examined. To obtain standardized quantitative results, external controls consisting of cDNA plasmid standards were constructed.

Statistical analysis

Data were expressed as mean ± SE. Statistic significance was examined with Wilcoxon signed-rank test and Student's *t* test, respectively. Probability below 0.05 ($P < 0.05$) was considered as significant.

RESULTS

The subcellular localization of endogenous and exogenous RhoA in SGC-7901 cells

With immunofluorescence staining (Figure 1) and Western blotting (Figure 2) endogenous RhoA was detected in the membrane, the cytosol, and the nucleus of SGC-7901 cells. As shown by Western blotting (Figure 3) exogenous RhoA was also localized in the membrane, the cytosol, and the nucleus of SGC-7901 cells.

The existence of RhoA in nucleoli of SGC-7901 cells

Both immunofluorescence staining (Figure 4) and Western blotting (Figure 5) indicated the nucleolus as the precise localization of RhoA within SGC-7901 interphase nucleus.

The change of RhoA expression during cell cycle

In mitotic active Hela cells, Western blotting indicated that the expression of RhoA protein in interphase (Figure 6A) was higher than that in mitosis phase (Figure 6B).

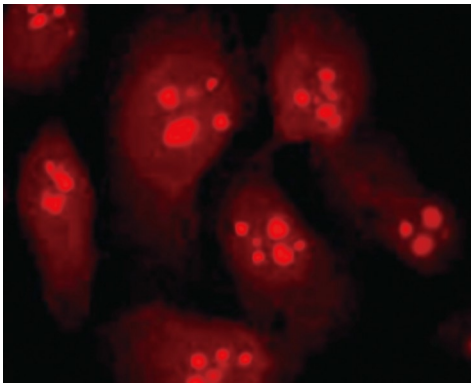


Figure 1 Localization of endogenous RhoA in SGC-7901 cells. RhoA was immunofluorescently stained with a specific mAb and it was localized in the cytoplasm, the membrane, and the nucleus (× 400).

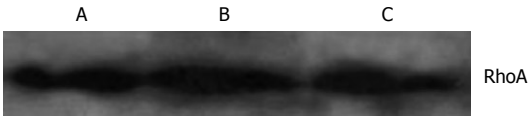


Figure 2 Distribution of RhoA in membrane, cytosol, and nucleus in SGC-7901 cells. SGC-7901 cells were lysed and membrane (A), cytosol (B) and nuclear (C) fractions were obtained. Expression of RhoA in each fraction was detected by Western blotting using a specific anti-RhoA mAb.

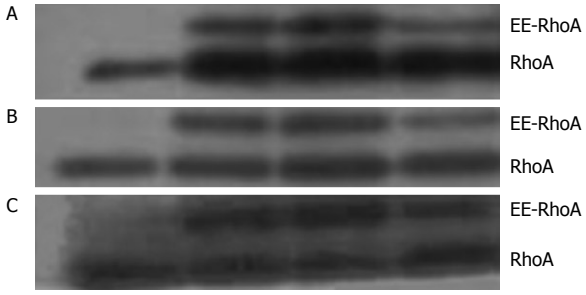


Figure 3 Distribution of endogenous and exogenous RhoA in membrane, cytosol, and nucleus in SGC-7901 cells. SGC-7901 cells were transfected with three types of exogenous EE-tagged RhoA plasmids, and 24 h later, cells were lysed and membrane (A), cytosolic (C) and nuclear (B) fractions were obtained. The expression of different RhoA proteins (lane 1 mock, lane 2 wild type, lane 3 63L, lane 4 N19) in each fraction was detected by Western blotting with mAb against RhoA.

Consistent with this finding, the amounts of RhoA transcripts were also found to be higher in interphase (Figure 7A) than in mitosis phase (Figure 7B).

LPA-induced membrane and nucleus translocation of RhoA in SGC-7901 cells

As shown by Western blotting, a stimulation with 1 μmol/L LPA at 37℃ for 15 min causes an increase in the amount of RhoA in the membrane (Figure 8A) and the nuclear (Figure 8B), but a decrease in the cytosol fraction (Figure 8C).

CPT-cAMP-induced cytosol translocation of RhoA in SGC-7901 cells

An incubation with 100 μmol/L cAMP at 37℃ for 30 min led to a decrease in the amounts of RhoA protein in membrane (Figure 9A) and nuclear (Figure 9B) fractions, but to an increase in cytosol preparations (Figure 9C).

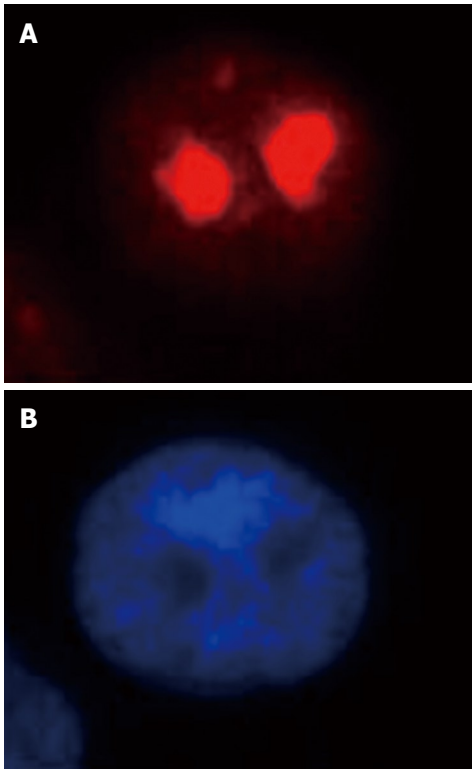


Figure 4 Localization of RhoA in the nucleolus of interphase SGC-7901 cells. RhoA in SGC-7901 cells of interphase was immunofluorescently stained with monoclonal antibody against RhoA (**A**). The chromosomes were stained with Hoechst 33342 (**B**) (× 1000).

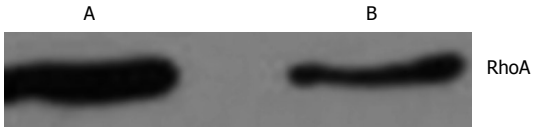


Figure 5 RhoA could be detected in nucleolus in SGC-7901 cells. SGC-7901 cells were lysed and the nucleolus fraction was obtained. Expression of RhoA in the nucleolus fraction (B) was detected by Western blotting with a mAb directed against RhoA, and total protein obtained from SGC-7901 cells (A) served as the positive control.

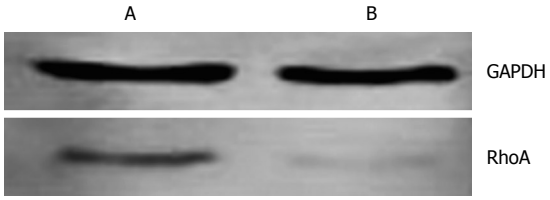


Figure 6 The expression of RhoA protein was higher in interphase than in mitosis phase in HeLa cells. HeLa cells were treated with Taxol (5 μg/mL, 12 h). Control (attached) (A) or mitotic ("shake off") (B) cells were lysed and the expressions of RhoA and GAPDH were detected by Western blotting with mABs directed against RhoA and GAPDH.

CPT-cAMP could block LPA-induced RhoA translocation in SGC-7901 cells

Stimulation with 100 μmol/L cAMP at 37℃ for 30 min

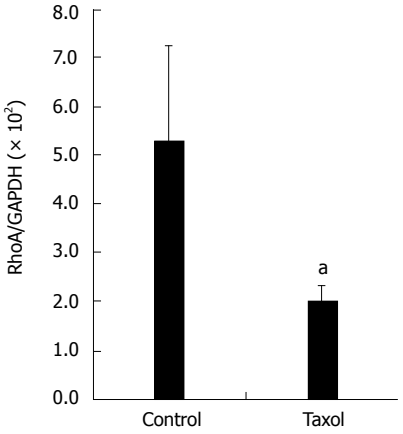


Figure 7 The mRNA expression of RhoA gene was higher in interphase than in mitosis phase in HeLa cells. HeLa cells were treated with Taxol (5 μg/mL, 12 h). Total mRNA was isolated from control (attached) and mitotic ("shake off") cells, and the expression of RhoA gene was detected by real-time quantitative PCR. Each bar represents mean ± SE obtained from five independent experiments. $^aP < 0.05$.

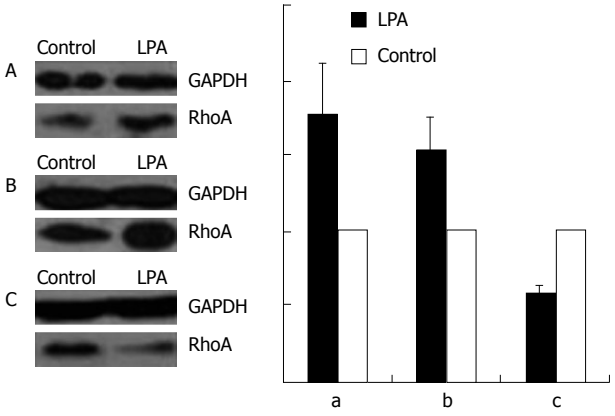


Figure 8 LPA-induced RhoA translocation from cytosol towards membrane and nucleus in SGC-7901 cells. SGC-7901 cells were treated with or without LPA (1 μmol/L, 15 min). Control or LPA -treated cells were lysed and the membrane (A), cytosolic (C), and nuclear (B) fractions were obtained. Expressions of RhoA and GAPDH proteins in each fraction were detected by Western blotting, and the representative band images were shown in the left panels. Densitometric analysis of the bands was shown in the right panels. Each bar represents mean ± SE obtained from five independent experiments. $^aP < 0.05$.

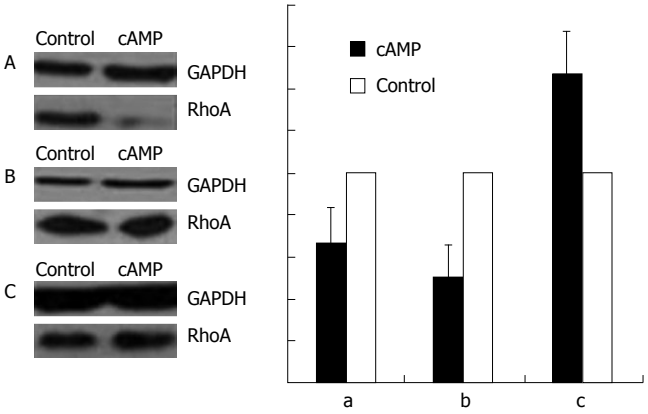


Figure 9 cAMP-induced RhoA translocation from membrane and nucleus towards cytosol in SGC-7901 cells. SGC-7901 cells were treated with or without CPT-cAMP (250 μmol/L, 30 min). Control or CPT-cAMP -treated cells were lysed, and membrane (A), cytosolic (C), and nuclear (B) fractions were obtained. Expressions of RhoA and GAPDH proteins in each fraction were detected by Western blotting, and the representative band images were shown in the left panels. Densitometric analysis of the bands was shown in the right panels. Each bar represents mean ± SE obtained from five independent experiments. $^aP < 0.05$.

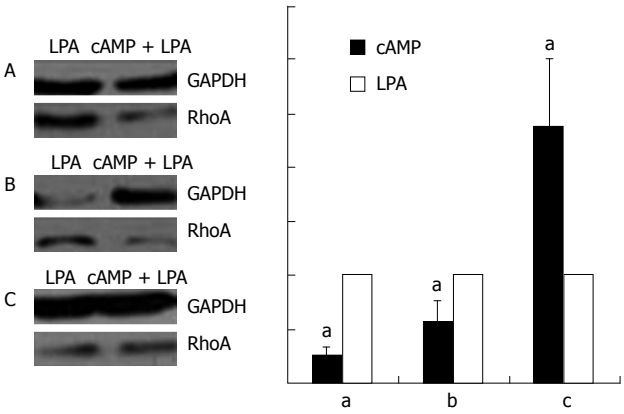


Figure 10 cAMP could block LPA-induced RhoA translocation in SGC-7901 cells. SGC-7901 cells were pretreated with CPT-cAMP (250 μmol/L, 30 min), and then with or without LPA (1 μmol/L, 15 min) treatment. LPA or CPT-cAMP+LPA -treated cells were lysed and membrane (A), cytosolic (C), and nuclear (B) fractions were obtained. Expressions of RhoA and GAPDH proteins in each fraction were detected by Western blotting, and the representative band images were shown in the left panels. Densitometric analysis of the bands was shown in the right panels. Each bar represents mean ± SE obtained from five independent experiments. $^aP < 0.05$.

followed by an incubation with 1 μmol/L LPA at 37℃ for 15 min was found to cause a decrease in the amounts of RhoA protein in the membrane (Figure 10A) and the nuclear (Figure 10B), but an increase in the cytosol (Figure 10C) preparations.

DISCUSSION

RhoA acts as a molecular switch in the cells, regulates signal transduction from cell surface receptors to intracellular target molecules, and is involved in a variety of biological processes, including cell morphology[25], motility[26], cytokinesis[27,28], smooth muscle contraction[29,30], and tumor progression[31,32]. It was widely accepted that the subcellular localization of RhoA was in the membrane and cytosol, and mainly in the latter one[33,34]. Recently, our laboratory reported the existence of RhoA in the nuclei of SGC-7901 cells[22]. In this study, we not only confirm the nuclear localization of RhoA; we were able to localize RhoA proteins more precisely, namely within the nucleolus.

Protein import into the cell nucleus is a typical transport process between the cytosol and the nucleus. It occurs through nuclear pore complexes (NPCs)[35-39]. These elaborate proteinaceous structures act not only as molecular sieves, allowing free diffusion of ions and small molecules, but also mediate the active transport of proteins and ribonucleoprotein particles. In order to enter the nucleus, proteins larger than about 60 kDa generally require a specific nuclear localization signal (NLS). Protein molecules can bind with transporting protein such as

importin *via* NLS and get into the nucleus through ATP dependent process. However, the molecular mass of RhoA is 21 kDa and there is no NLS with its protein structure. Our primary experiment did not detect the combination of RhoA with nuclear transporting proteins such as importin α and importin β (data not shown). So, it was reasonable for us to presume that the molecular mechanism of RhoA protein transportation to the nucleus was not through the classic transportation pathway, but diffusion.

The nucleolus of eukaryotic cells was first described in the early 19th century and was discovered in the 1960s to be the site of ribosome synthesis. It owned a variety of roles of cell activity. rRNA transcription, rRNA processing, and ribosome assembly have been clearly established as major functions of the nucleolus. As research progresses, many other functions of the nucleolus have been recognized, including gene expression, processing of nuclear export of certain mRNAs, processing of U6 RNA, one of the spliceosomal small nuclear RNAs, and processing of tRNA precursors. Recently, the plurifunctional nucleolus concept has been widely accepted.

According to the concept of plurifunctional nucleolus, it is reasonable to speculate that RhoA will share, at least, part of the function of the nucleolus. A previous study suggested a role for major nucleolar proteins in the nucleocytoplasmic transport of ribosomal components, and transient exposure of shuttling proteins to the cytoplasm may provide a mechanism for cytoplasmic regulation of nuclear activities[40]. The expression of some nucleolar proteins, such as B23, changes during cell cycle. In this experiment, the results showed that the amount RhoA protein also changed during cell cycle, exhibiting high amount in S phase, and the change of RhoA mRNA expression was the same as that of the protein. This suggested that RhoA probably played a role in regulation of cell mitogenesis. In this way, our results obviously provide a new insight into investigating some unknown functions of RhoA according to its new distribution.

In our previous study[22], with immunofluorescent microscopy, we did not detect significant translocation of RhoA in SGC-790 treated with RhoA activator LPA or RhoA activation inhibiting reagent cAMP. Since some other studies reported RhoA translocation according to its activity with Western blot assay, in this experiment, we applied different methods to separate cellular components and western blotting to investigate RhoA translocation, especially in nucleus. With these more concise and sensitive methods, we showed that after the treatments with LPA, cAMP, and cAMP + LPA, nucleus translocation of RhoA was the same as its membrane translocation. Furthermore, since it was reported that RhoA in membrane represented its active form[41] and our results showed that RhoA moved into the nucleus and onto the membrane during the stimulation of LPA, we supposed that RhoA in nucleus is also in the active status.

In conclusion, our results indicated that the nucleolus provided a new compartment for RhoA in SGC-7901 cells and that the activation of RhoA had an obvious effect on its translocation, stimulating the distribution of this protein not only in membrane but also in nucleus.

COMMENTS

Background

Until now, more than 100 small G proteins have been found. Among them, only Ran/TC4, Rap1, and H-Ras were found to be located within the nucleus. However, the nuclear localization of these small G proteins was closely related to the proliferation and the malignant change of the cells.

Research frontiers

Both Rap1 and H-Ras are members of Ras family. Active Rap1A and Rap1B were located within the nucleus of epithelium-originated cancer cells, and the distribution was related to the malignancy of the cells. Active H-Ras was found to be located within the nucleus of both transformed cells and NDEA-induced hepatic tumor cells of mice.

Innovations and breakthroughs

Through immunofluorescence microscopy and Western blotting, RhoA has been found to be located in the nucleus of the gastric cancer cell line SGC-7901. The phenomenon was confirmed by confocal microscope observation of vital cells transfected with plasmid encoding GFP-tagged RhoA. However, the concise localization of the protein within the nucleus was not clear. Furthermore, whether the activation of RhoA is related to its nuclear localization or not was not elucidated.

Applications

Since RhoA was found to be located on the nucleolus of the cells and some nucleolar proteins played important role in controlling cell mitosis, it deserves to study the role of nucleolar RhoA in controlling cell mitosis.

Terminology

Small G proteins are GTP/GDP binding proteins with molecular size around 20 kD. Each small G protein has two forms: GTP-binding active form and GDP-binding inactive form. When it changes from GDP-binding form to GTP-binding form, it is activated. Through this change cycle, small G protein performs as molecular switch. RhoA is an important member of Rho small G protein family. It has been found that RhoA played an important role in regulating diverse biological activities of cells.

Peer review

The conclusion of this study was as follows: The localization of RhoA in the SGC-7901 cells was in membrane, cytosol, and nucleus. The novel finding of this study is showing that the precise localization of RhoA protein was in the nucleolus.

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LIVER CANCER

Prokineticin 2/Bv8 is expressed in Kupffer cells in liver and is down regulated in human hepatocellular carcinoma

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Abstract

AIM: To study the implication of prokineticin 1 (PK1/EG-VEGF) and prokineticin 2 (PK2/Bv8) in hepatocellular carcinoma angiogenesis.

METHODS: The gene induction of PK1/EG-VEGF and PK2/Bv8 was investigated in 10 normal, 28 fibrotic and 28 tumoral livers by using real time PCR. Their expression was compared to the expression of VEGF (an angiogenesis marker), vWF (an endothelial cell marker) and to CD68 (a monocyte/macrophage marker). Furthermore, the mRNA levels of PK1/EG-VEGF, PK2/Bv8, prokineticin receptor 1 and 2 were evaluated by real time PCR in isolated liver cell populations. Finally, PK2/Bv8 protein was detected in normal liver paraffin sections and in isolated liver cells by immunohistochemistry and immunocytochemistry.

RESULTS: PK2/Bv8 mRNA but not PK1/EG-VEGF was expressed in all types of normal liver samples examined. In the context of liver tumor development, we reported that PK2/Bv8 correlates only with CD68 and showed a significant decrease in expression as the pathology evolves towards cancer. Whereas, VEGF and vWF mRNA were significantly upregulated in both fibrosis and HCC, as expected. In addition, out of all isolated liver cells examined, only Kupffer cells (liver resident macrophages) express significant levels of PK2/Bv8 and its receptors, prokineticin receptor 1 and 2.

CONCLUSION: In normal liver PK2/Bv8 and its receptors were specifically expressed by Kupffer cells. PK2/Bv8 expression decreased as the liver evolves towards cancer and did not correlate with HCC angiogenesis.

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Key words: Prokineticin; Hepatocellular carcinoma; PK2/Bv8; Angiogenesis; Kupffer cells; Vascular endothelial growth factor; Liver

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INTRODUCTION

In vivo, a solid tumor can not progress beyond a few cubic millimeters without oxygen and nutrients supplied by an adequate vascular support[1]. The creation of novel vascular vessels in the tumor microenvironment resulting from an over expression of pro-angiogenic factors plays a central role in tumor invasion and metastasis[2]. Hepatocellular carcinoma (HCC) present typical neo vascularization[3]. Among the soluble factors responsible for the shift towards tumor neo vascularization, vascular endothelial growth factor (VEGF) is thought to play an important role in HCC. This factor is synthesized essentially during hypoxic stress[4] and promotes the growth, migration, morphogenesis of vascular endothelial cells and increases vascular permeability[5,6]. Furthermore, the VEGF/VEGF receptor system is thought to be closely related to the histological grade of hepatocellular carcinoma[7] and strongly expressed in well differentiated HCC[8]. Several other potent angiogenic factors have been reported to be

involved in HCC angiogenesis, notably basic fibroblast growth factor (bFGF)[9,10], angiopoietin 1 (Ang1) and angiopoietin 2 (Ang2)[11]. Interestingly, inflammatory cytokines like Interleukin 8 (IL-8) and tumor necrosis factor-alpha (TNF-α) have also been shown to be implicated in the maintenance of HCC angiogenesis but also in chronic hepatitis[12,13].

Prokineticin 1, also named endocrine gland-vascular endothelial growth factor (PK1/EG-VEGF) and prokineticin 2 also known as Bombina variegata 8 (PK2/Bv8) are widely distributed in mammalian tissue and exhibit diverse biological activities. Prokineticins bioactivities includes, smooth muscle contraction in the gastrointestinal tract[14], supporting neuronal survival, pain sensation and circadian rhythms in the central nervous system[15-17]. Prokineticins display also potent cytokine properties, such as inducing bone marrow leukocyte production, and modulating the growth and function of peripheral leukocytes[18-20]. These peptides were also shown to act as angiogenic mitogens, indeed, PK1/EG-VEGF and PK2/Bv8 induce proliferation, migration and fenestration of endothelial cells from adrenal capillaries[21,22]. In addition, in a mouse model of myocardial infarctions, PK2/Bv8 protected cardiomyocytes by increasing neovascularization[23]. Prokineticins are a family of peptides characterized by a common AVIT motif, and 10 conserved cysteine residues[24-26]. Prokineticins are not structurally related to VEGF and mediate their biological activity by selectively acting on two G protein coupled receptors, prokineticin receptor 1 and 2 (PKR1 and PKR2), for which they have similar affinities[24,26]. Activation of PKRs triggers multiple signaling pathways, such as calcium mobilization, phosphoinositol turnover, and activation of Akt kinase and mitogen-activated protein kinase (MAPK)[27].

Numerous studies have evaluated the implication of prokineticins in tumor development. For example, mice implanted with colorectal cancer cell line SW620 stably expressing PK1/EG-VEGF had an increase of angiogenesis and of colorectal cancer metastasis[28]. In ovarian carcinoma, PK1/EG-VEGF was evenly detected in benign, low malignant potential neoplasms or stage I ovarian cancer[29], and no expression was observed in endometrial carcinoma[30]. However, PK1/EG-VEGF was shown to be associated in Leydig cell tumor growth[31] and in neuroblastoma progression[32]. In prostate cancer, both prokineticin 1 and 2 and their receptors were up regulated[32,33]. Taken altogether, the literature indicates that prokineticins are differentially associated to tumor development. In the present study, we explored the potential implication of both prokineticins in HCC angiogenesis, and identified their cellular origin in liver parenchyma.

MATERIALS AND METHODS

Patients

Liver tissue samples were obtained from 28 patients with HCC undergoing surgical resection of the tumor. Patients with HCC were 25 males and 3 females (59.9 ± 9.0 years). The etiology of fibrosis (n = 8) and cirrhosis (n = 20) included hepatitis C (n = 22), hepatitis B (n = 7) and alcohol abuse (n = 14). All tumor samples were taken within the tumor, and only tissue with anatomic pathology features that allow a matching diagnosis with the pathology report of each patient were used for RNA extraction. Matching non tumor livers (n = 28) were taken distantly from the tumors. Histological evaluation before their inclusion in the study ruled out the presence of tumor tissue in these samples. Controls were 10 histologically normal liver samples obtained from metastatic livers of colorectal cancer[34]. Access to this material was in agreement with French laws and satisfied the requirements of the local Ethics Committee. The histological stage of fibrosis and the intensity of necroinflammatory lesions were graded according to the METAVIR score[35]: A0 = no activity, A1 = mild, A2 = moderate, A3 = severe, F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with few septa, F3 = numerous septa without cirrhosis, and F4 = cirrhosis. All tissue sections were routinely analyzed after staining with hematoxylin-eosin-saffran and Sirius red.

Human hepatic cell isolation

Human hepatic cells were isolated from histologically normal specimens of partial hepatectomy from patients undergoing hepatic resection for liver metastases. A2 step perfusion method using collagenase and pronase, followed by differential sedimentation allowed separation and recovery of hepatocytes (HA) from non-parenchymal cells enriched in sinusoidal cells[36]. From the latter fraction, hepatic stellate cells (HSC) were efficiently purified by a density gradient centrifugation with Nycodenz 13% (Sigma-Aldrich, St. Louis, MO) and cultured as previously described[37]. Similarly a population enriched in liver macrophages, also known as Kupffer cells, was obtained from the same non-parenchymal cell population by using a density gradient centrifugation with Nycodenz 17%[38]. The proportion of Kupffer cells present in the Kupffer cell enriched population (KC-Enr) was evaluated by flow cytometry (Becton Dickinson's FACScalibur, Grenoble, France) using an antibody directed against CD14, a typical surface marker of monocyte/macrophage (Immunotech Beckman Coulter, Marseille, France). Kupffer cells were then further enriched from the Kupffer cell enriched population (KC-Enr) by using anti-CD14-Dynabeads according to the manufacturer's indication (Dynal A.S., Oslo, Norway)[38].

Hepatoma cell lines (Hep3B, BC1, B16A2) were cultured in 25% 199, 75% MEM medium (Eurobio, Courtaboeuf, France), supplemented with 1 mg/mL albumin, 5 μg/mL insulin, 50 000 U/L penicillin-streptomycin, 12.5 nmol/L glutamine and 100 ng/mL hydrocortisone. HepG2 cell line was cultivated in MEM α Medium (GIBCO, Paisley, Scotland), to which was added 50 000 U/L penicillin-streptomycin and 12.5 nmol/L glutamine.

RNA isolation and RT-PCR

Total RNA was extracted from isolated human liver cells, liver tissue or directly from Kupffer cells linked to the

Table 1 Primer sequences for quantitative PCR

Gene		Primer sequence	Primer sequence %
VEGF	sense	AGGAGGAGGGCAGAATCATCA	99
	antisense	CTCGATTGGATGGCAGTAGCT	
vWF	sense	TGGAGCAGCAAAGGGACGAGA	99
	antisense	TAGGAGGAGGGGCTTCAGGGG	
PK2/Bv8	sense	TACAGCTTTTGGTCCCTTGC	97
	antisense	GATTCCCATCAGTGATTCTGC	
CD68	sense	TAGCTGGACTTTGGGTGAGG	99
	antisense	AAGGATGGCAGCAAAGTAGC	
PKR1	sense	GCTCATGTGCATCCTCACC	99
	antisense	GATCATGCTGTTGCTCAGG	
PKR2	sense	CTTCTTCCCCACTGTGTTCG	99
	antisense	GGTGTTGATCATGCTGTTGC	
EG-VEGF	sense	GGGCTTCAGTGGTTAACTGG	97
	antisense	TGTGACCTGTGACCTTCTGC	
18S	sense	CCATCCAATCGGTAGTAGCG	98
	antisense	GTAACCCGTGGAACCCCATT	
β-actin	sense	GATGAGATTGGCATGGCTTT	95
	antisense	GAGAAGTGGGGTGGCTT	

CD14 coated beads using the SV Total RNA isolation Kit® (Promega, Charbonnieres-lesbains, France) and 1 μg of total extracted RNA was subjected to a reverse transcription reaction using high capacity cDNA archive kit® (Applied Biosystem, Foster City, CA). A total of 12.5 ng total complementary DNA was used as a template for amplification with primers specific for PK1/EG-VEGF, PK2/Bv8 (PK2β and PK2L) and β-actin (Table 1) used at a 250 nmol/L final concentration. The PCR reaction performed using PCR master mix from Promega for which the final working concentration included 0.5 units of Taq polymerase, 200 μmol/L dNTPs and 1.5 mmol/L $MgCl_2$. The PCR reaction was performed using GeneAMP PCR 9700 (Applied Biosystems), after an initial denaturation step at 94℃ for 2 min, 35 cycles of PCR were programmed for PK1/EG-VEGF and PK2/Bv8, whereas 25 cycles were performed for β-actin. Denaturation step was performed at 94℃ for 30 s, annealing at 60℃ for PK1/EG-VEGF and PK2/Bv8, and 55℃ for β-actin, and extension at 72℃ for 1 min. The last extension was at 72℃ for 7 min.

For real-time quantitative PCR, the mRNA levels of PK1/EG-VEGF, PK2/Bv8 (PK2β and PK2L), VEGF (VEGF A), vWF, CD68, PKR1 and PKR2 were assayed using the 7000 sequence detection system ABI Prism® sequence detector (Applied Biosystems), using the double strand specific dye SYBR® Green system (Applied Biosystems). Primer sequences are summarized in Table 1. The PCR condition and cycle were as follows: initial DNA denaturation 10 min at 95℃, followed by 40 cycles of denaturation at 95℃ for 15 sec, follow by an annealing step, and then extension at 60℃ during 1 min. Each point was performed in triplicate. To ensure that the primers produced a single and specific PCR amplification product, a dissociation curve was performed during the PCR cycle and only primers with a unique dissociation peak were selected, followed by migration on a 2% agarose gel to ensure that the PCR product was unique. The PCR products were then sequenced to confirm that the correct cDNA was amplified (data not shown). The amplification efficiency for each primer pair was calculated and presented in Table 1. The expression level of each gene was adjusted by the level of 18S mRNA and expressed as the ratio to 18S mRNA or as the ratio to the average gene expression level in normal liver.

Immunohistochemical staining

Paraffin embedded liver sections were de-parafinised by 2 changes of xylene for 5 min, and re hydrated, in 2 changes of 100% ethanol for 3 min, 2 changes of 95% and 80% for 1 min, and then washed with distilled water. Antigen retrieval was performed using a citrate pH 6.0 buffer, heated at 95℃-100℃ for 40 min; sections were let to rest at room temperature for 20 min. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide (10 min, RT) and the non specific sites were blocked with PBS/BSA 2% (1 h, RT). PK2/Bv8 protein was detected using rabbit polyclonal anti-PK2/Bv8 antibody (gift of Dr. Feige, INSERM U858, Grenoble, France) at a final concentration of 1.44 μg/mL for 1 h at RT or for 24 h at 4℃[39]. CD31, CD34, CD68 and VEGF detection was performed by incubating sections with primary antibodies overnight at 4℃ using mouse monoclonal anti-CD31 IgG1 (clone JC70A, dilution 1:50, Dako, Glostrup, Denmark), mouse monoclonal anti-CD34 IgG1 (clone Qbend10, dilution 1:10 Immunotech, Beckman Coulter), mouse monoclonal anti-CD68 IgG3 (clone PGM1, ready to use, Dako), rabbit polyclonal anti-VEGF (sc-152, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were diluted in Dako's REAL™ Antibody Diluent. Sections were processed with appropriate biotinylated secondary antibody and a streptavidin biotin peroxidase amplification kit (Vectastain, Vector Laboratories, Burlingame, CA). The peroxidase reaction was finally developed with diaminobenzidine (Merck, Whitehouse Station, NJ) and sections were counter stained with Mayer's hematoxylin. Non-immune rabbit serum or control mouse IgG was used instead of primary antibodies as negative controls. In addition, competition experiments were performed by pre-incubating overnight at 4℃ PK2/Bv8 antibodies with the appropriate antigen peptides.

Immunocytochemical staining

Enriched Kupffer cells (10^5 cells) were spotted on slides by cytospin (10 min at 400 rpm) and then fixed using 4% paraformaldehyde at room temperature for 10 min. Permeabilization was achieved by treating cells for 10 min at RT with Triton × 100 0.1% and blocking of non-specific sites by 20min incubation at RT with PBS/BSA 2%. PK2/Bv8 protein was detected with mouse anti-PK2/Bv8 anti-serum (dilution 1:50, Abnova, Tapei City, Taiwan) and revealed by TRITC labeled anti-mouse antibody (dilution 1:150, Merck). CD68 protein was detected directly with FITC labeled mouse monoclonal anti-CD68 IgG1 (clone KP1, dilution 1:10, Dako). Primary and secondary antibodies were diluted in appropriate diluent (Dako's REAL™ Antibody Diluent) and incubated at RT for 2 h and 1 h respectively. The cells were counterstained with nucleus dye DAPI (dilution 1:10000, Dako). Sections processed with one or both of the control primary antibodies (control mouse

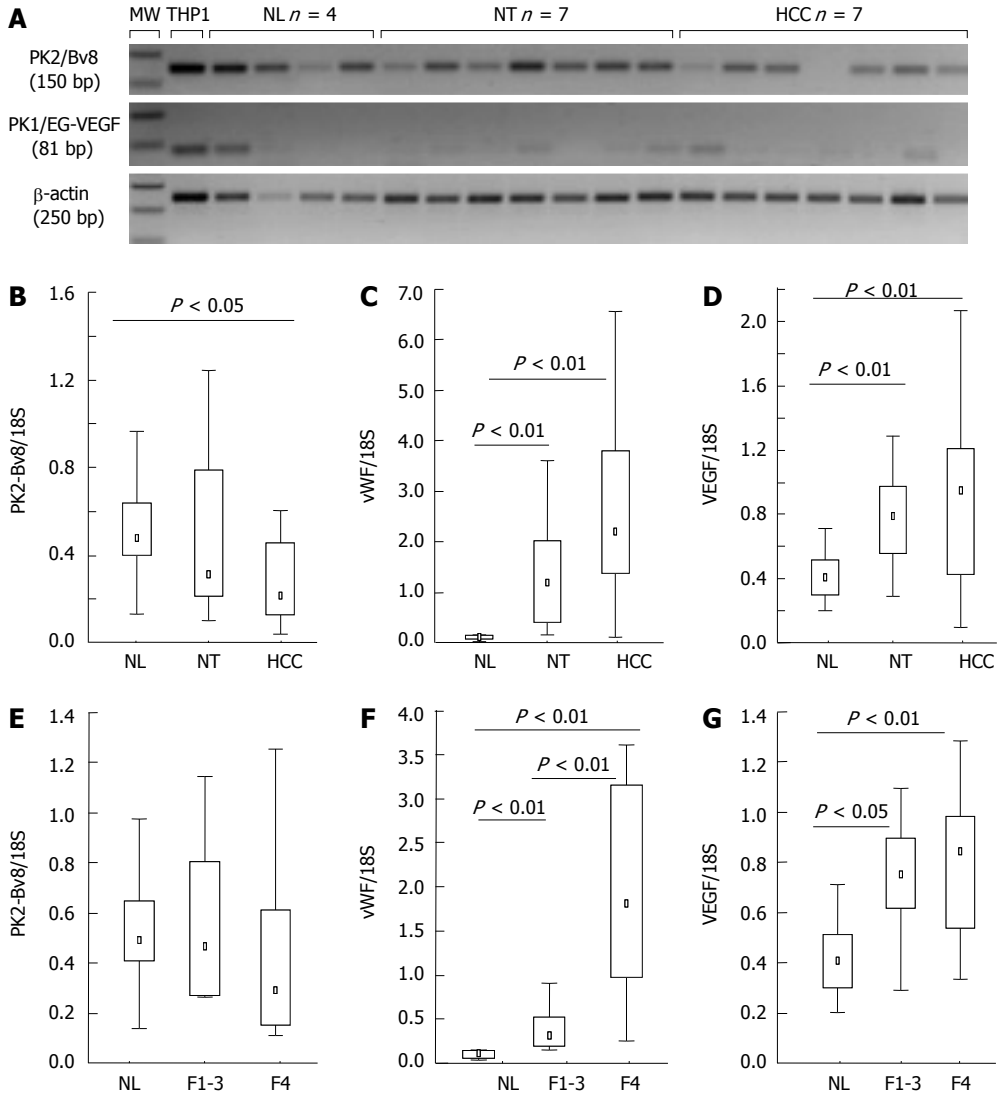


Figure 1 (**A**) RT-PCR analysis of PK1/EG-VEGF and PK2/Bv8, and (**B-G**), real-time PCR analysis of PK2/Bv8, vWF and VEGF in normal liver (NL, $n = 10$), non-tumorous liver (NT, $n = 28$), and in Hepatocellular carcinoma (HCC, $n = 28$).

serum and/or control IgG1) were free of immunostaining in their corresponding color channel. We checked that FITC labeled cells remained negative in TRITC channel.

Statistical analysis

Statistical comparisons for significance were made with Wilcoxon's test for paired samples, and with Mann-Whitney *U* test for un-paired samples. The correlation between continuous variables was examined by means of Spearman's rank-order coefficients. A level of $P < 0.05$ was considered significant. Calculations were made with the commercially available software Statistical.

RESULTS

Expression of PK1/EG-VEGF, PK2/Bv8, vWF and VEGF mRNA in normal and tumoral livers

The mRNA expression of PK1/EG-VEGF and PK2/Bv8 in liver was first evaluated by PCR in 4 normal livers (NL), 7 tumoral livers (HCC) and in their corresponding adjacent non-tumoral tissue (NT) (Figure 1A). THP1, a monocytic cell line which expresses high levels of both PK1/EG-VEGF and PK2/Bv8 transcripts, served as a positive control. Our results show that, in all liver samples analyzed PK2/Bv8 is significantly expressed, whereas PK1/EG-VEGF is barely detectable.

In order to monitor more precisely how PK2/Bv8 varied during HCC progression, we measured the mRNA expression of PK2/Bv8 by real time PCR in a larger cohort of 28 patients (Figure 1B). PK2/Bv8 mRNA is expressed in normal liver ($n = 10$) at significant levels (average cycle threshold is approximately 28). However, a significant decrease in PK2/Bv8 mRNA expression was detected in HCC relative to normal liver tissues ($P < 0.05$), while no significant difference was found between HCC ($n = 28$) and peri-carcinomatous liver tissues (NT, $n = 28$). Such expression of PK2/Bv8 was compared to two angiogenesis markers vWF (Figure 1C) and VEGF (Figure 1D). vWF, an endothelial cell marker, showed remarkable over expression in tumor ($P < 0.01$) and in adjacent liver tissues compared with normal liver ($P < 0.01$). Similarly, VEGF mRNA levels were induced in HCC and the underlying cirrhotic liver (NT) compared with normal liver ($P < 0.01$). By using the spearman's rank order correlation, a significant correlation was observed between VEGF and vWF ($r = 0.6$; $P < 0.001$) but not with PK2/Bv8.

The peri-carcinomatous liver tissues were classified according to the METAVIR score. vWF and VEGF were

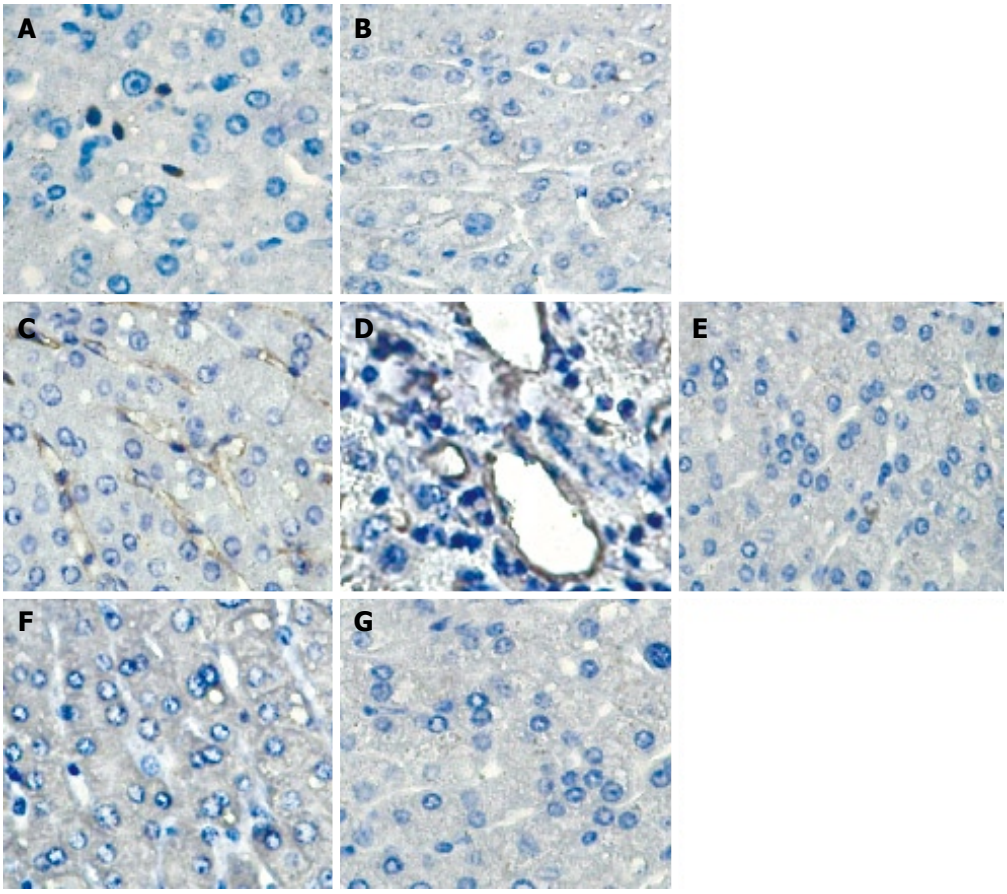


Figure 2 Immunolocalization of PK2/Bv8, CD31, CD34 and VEGF on normal liver sections. **A**: Bv8; **B**: Bv8 + blocking peptide; **C**: CD31; **D**: CD34; **E**: Mouse IgG1; **F**: VEGF; **G**: Rabbit IgG.

significantly up-regulated in fibrosis and cirrhosis ($P < 0.01$) compared with normal liver (Figure 1F and G) whereas no difference was observed with PK2/Bv8 (Figure 1E). Moreover, PK2/Bv8, vWF and VEGF mRNA expression did not show any significant statistical correlation with necro-inflammatory activity (data not shown).

Immunolocalization of PK2/Bv8, CD31, CD34 and VEGF in normal livers

Sections of normal livers from 5 different individuals were immunostained with antibodies against: PK2/Bv8, CD31 (a constitutive marker of endothelial cells), CD34 (a marker of endothelial cell in proliferation), and with an antibody against VEGF A (including 165, 189 and 121 variants). PK2/Bv8 protein expression was detected in cells restricted to the hepatic sinusoids and presenting morphology similar to resident macrophages (Figure 2). The specificity of the staining was demonstrated by the inhibition of PK2/Bv8 labeling after anti-PK2/Bv8 pre-incubation with an excess of recombinant PK2/Bv8 peptide. In normal liver, both large capillary endothelial cells and sinusoidal endothelial cells were positive for CD31, whereas CD34 positive endothelial cells were practically un-detectable in the parenchyma, excepted in large capillaries. VEGF protein was detected at low levels in the hepatic parenchyma.

Expression of CD68 mRNA and protein in normal and tumoral livers

Recent reports have demonstrated that PK2/Bv8 has cytokine properties[20] and is strongly expressed by several types of leukocytes including the monocyte/macrophage lineage[19]. We therefore, hypothesized that the liver resident macrophages might be responsible for the expression of PK2/Bv8. The Kupffer cell population expresses the specific marker CD68, and on this basis we investigated the correlation between CD68 and PK2/Bv8 in term of transcripts and tissue localization. The mRNA expression of CD68 was measured by quantitative PCR in the same cohort of patients. As shown in Figure 3A, there was a significant down expression of CD68 in liver HCCs compared to the expression in normal livers ($P < 0.05$). Thus the expressions of PK2/Bv8 (Figure 1A) and CD68 (Figure 3A) in HCC patients follow a similar down regulation pattern in tumors relative to control livers. Furthermore, using spearman's rank order correlation, CD68 showed a correlation with PK2/Bv8 ($r = 0.45$, $P < 0.01$). Similar to PK2/Bv8, CD68 did not show any correlation of expression with VEGF or vWF. In addition, CD68 and PK2/Bv8 were immunolocalized in normal liver serial sections (Figure 3B). The specificity of PK2/Bv8 staining was evaluated by the disappearance of labeling when anti-PK2/Bv8 antibodies were pre-incubated with antigen peptide. We showed that most CD68 positive cells were not PK2/Bv8 positive. However, all cells showing positive staining for PK2/Bv8 were positive for CD68 staining.

Expression of PK1/EG-VEGF and PK2/Bv8 in Kupffer cell enriched non-parenchymal cells and in isolated hepatic cells

To further investigate the relationship between Kupffer cell population and PK2/Bv8 positive cells in liver, we studied various sequentially purified liver hepatic cells: hepatocytes

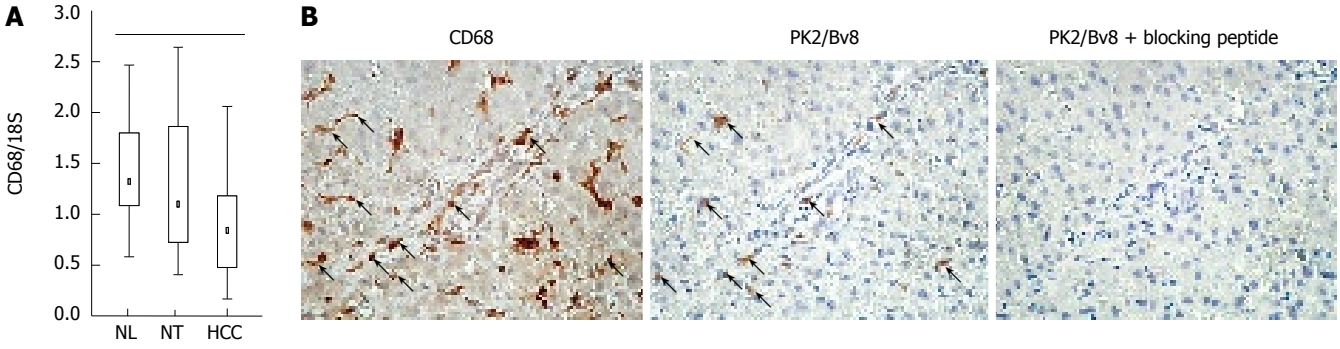


Figure 3 (**A**) Expression of CD68 normal liver (NL, *n* = 10), non-tumorous liver (NT, *n* = 28), and in Hepatocellular carcinoma (HCC, *n* = 28) by real time PCR, and (**B**) immunolocalization of CD68, PK2/Bv8, and PK2/Bv8 with blocking peptide on normal liver paraffin embedded serial sections.

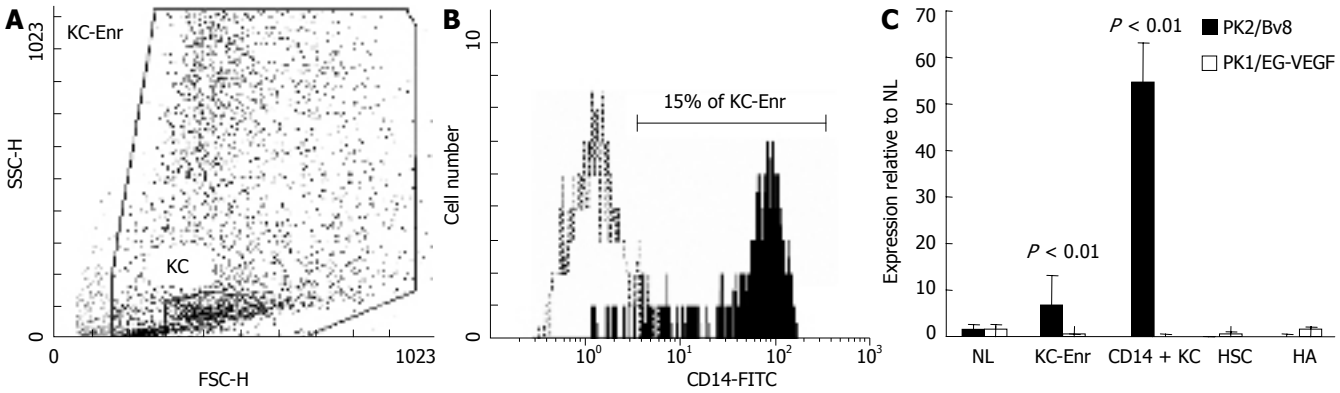


Figure 4 (**A**) Scatter plot and (**B**) histogram plot of CD14+enriched Kupffer cells, and (**C**) expression of PK1/EG-VEGF and PK2/Bv8 mRNA in isolated human liver cells by real time PCR.

(HA), hepatic stellate cells (HSC), and hepatic non parenchymal cell enriched in Kupffer cells (KC-Enr). The proportion of Kupffer cells in the KC-Enr population was evaluated by flow cytometry using an antibody against the cell surface monocyte/macrophage marker, CD14 (Figure 4A and B). This CD14 positive cell fraction represents 15% of the total KC-Enr population (representative of two separate experiments). CD14 positive Kupffer cells (CD14 + KC) were further enriched using anti-CD14 labeled microbeads.

The mRNA expression of PK1/EG-VEGF and PK2/Bv8 was measured by real time PCR on the various isolated liver cell populations, and compared whole liver expression. As shown in Figure 4C, the mRNA expression of PK2/Bv8 increases gradually as the concentration of Kupffer cells increases. Indeed in the KC-Enr cell population, containing 15% of Kupffer cells, the expression of PK2/Bv8 mRNA is 5 fold higher then in normal liver where Kupffer cells compose roughly 2% of the liver mass[40]. In CD14+ Kupffer cells (CD14+KC), expression of PK2/Bv8 is 35 fold higher than in normal liver. Primary hepatocytes, primary hepatic stellate showed no significant PK2/Bv8 expression (Figure 4C), as did several human hepatoma cell lines; BC1, Hep3B, HepG2, B16A2 (data not shown). Concerning PK1/EG-VEGF mRNA expression, it was barely detectable in all liver samples analyzed as shown in Figure 4C (average cycle threshold is about 33). All together these data strongly suggest that PK2/Bv8 transcript production in liver is associated with Kupffer cells.

Immunocytochemical co-localization of CD68 and PK2/Bv8 in isolated human liver cells

The human non-parenchymal liver cell population enriched in Kupffer cells (KC-Enr) was spotted on slides for a double Immunolocalization using FITC directly labeled anti-CD68 and indirectly labeled TRITC anti-PK2/Bv8. Results are presented in Figure 5, CD68 positive cells are shown in green and PK2/Bv8 positive cells appear in red, appropriate negative controls for both of the antibodies showed no specific staining. Co-immunolocalization of PK2/Bv8 and CD68 is shown in orange and demonstrates that all Bv8 positive cells are also CD68 positive.

Expression of PKR1 and PKR2 mRNA in human isolated liver cells and hepatoma cell lines

In order to assess the cellular target of PK2/Bv8 in liver cells, the mRNA level of their receptors, PKR1 and PKR2, was measured by real time PCR in isolated hepatic cells which includes CD14+ KC cells, hepatocytes, stellate cells, and human hepatoma cell lines BC1, Hep3B, HepG2, B16A2. In comparison to normal liver, PKR1 and PKR2 are most strongly expressed by the CD14 positive Kupffer cell population and expression was very weak in cultured stellate cells, in hepatoma cell lines and undetectable in hepatocytes (Figure 6).

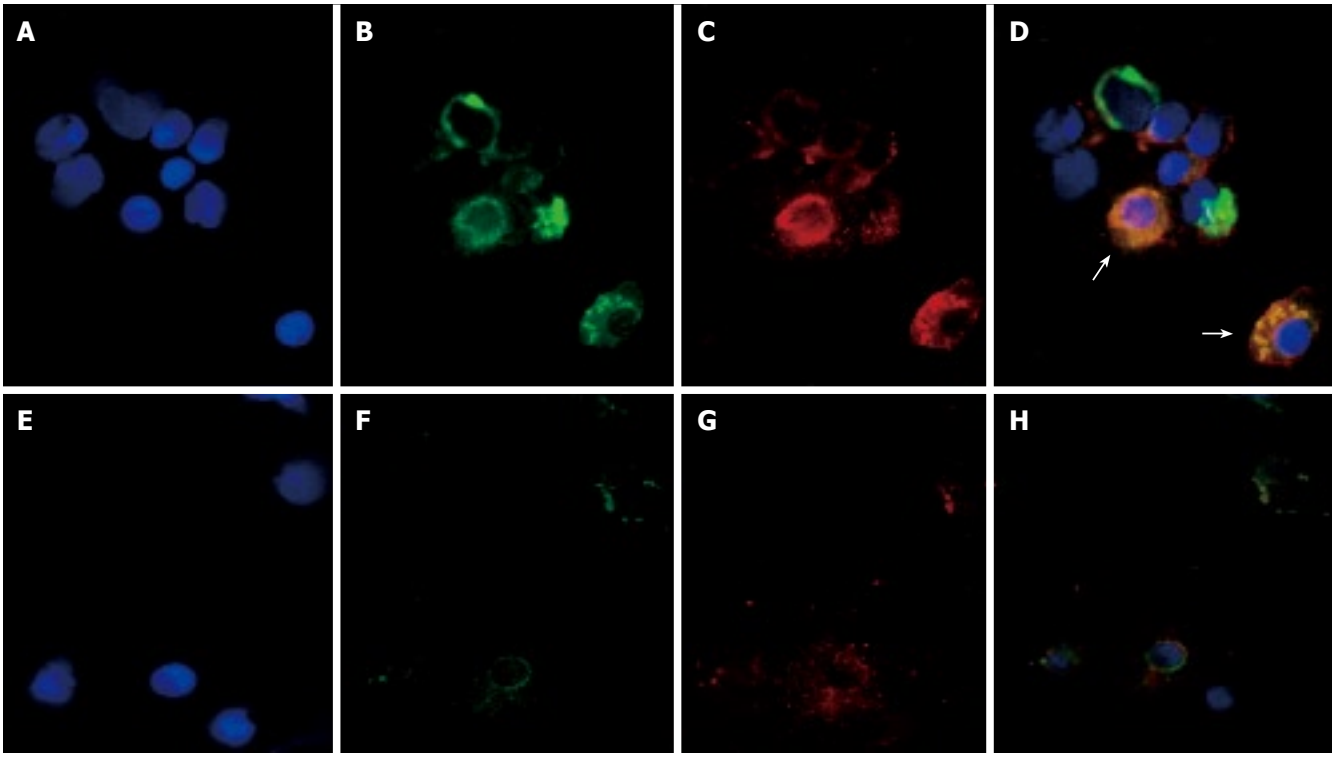


Figure 5 Immunocytochemical staining of PK2/Bv8 and CD68 on isolated liver cells enriched in CD14+ Kupffer cells. **A**: DAPI; **B**: CD68-FITC; **C**: Bv8/PK2-TRITC; **D**: Merge; **E**: DAPI; **F**: Mouse IgG1; **G**: Mouse serum; **H**: Merge.

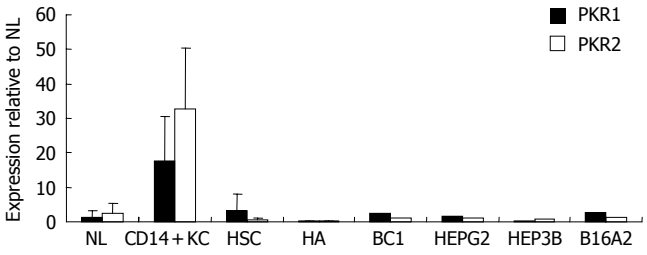


Figure 6 Expression of PKR1 and PKR2 mRNA in isolated human liver cells, by real time PCR.

DISCUSSION

PK1/EG-VEGF and PK2/Bv8 are two novel peptides with diverse biological functions[27,41] and one of such bioactivities is their ability to act as angiogenic mitogens. Their angiogenic activity was first evaluated in an *in vitro* assay by observing their capacity to induce proliferation, survival and migration of bovine adrenal cortical capillary endothelial cells (ACE)[21,22]. In this report we investigated the angiogenic potential of both PK1/EG-VEGF and PK2/Bv8 in human hepatocellular carcinoma (HCC). Interestingly, the literature shows two reports with divergent results on prokineticin expression in liver. In 2001, Li *et al*[14] showed an equivalent expression of prokineticin 1 and 2 in human liver by northern blot assay, whereas in 2005, Chen and colleagues[42], using RT-PCR observed a strong expression for PK2/Bv8 in human liver and an undetectable expression for PK1/EG-VEGF. Confirming the latter report, our results show a predominant expression of PK2/Bv8 in liver. Thus, we investigated further the expression of PK2/Bv8 in human liver, and its potential role in HCC angiogenesis. Our results show that Bv8/PK2 mRNA levels decrease significantly as the pathology evolves towards cancer.

Most cases of HCC develop on a liver fibrosis/cirrhosis background, which constitutes a very hypoxic environment for liver cells, and drives the production of numerous potent angiogenic factors[43]. In our HCC cohort, we monitored angiogenesis by measuring the expression of the endothelial cell marker; vWF and the angiogenic factor VEGF. Our results show that they are both strongly up-regulated in fibrosis and even more in tumors, confirming that our cohort exhibits a strong angiogenic profile. However, even though our HCC cohort constitutes an angiogenic environment, expression of PK2/Bv8 decreases in fibrosis and even more in tumors, suggesting that in this tissue context PK2/Bv8 is not induced by hypoxia in fibrosis and HCC.

To investigate further why PK2/Bv8, which is expressed at significant levels in normal liver, is down regulated through out the course of HCC development, we identified its cellular origin by several different techniques. Firstly, by immunolocalization on liver paraffin sections, PK2/Bv8 positive cells liver were located in the hepatic sinusoids and showed morphology similar to resident macrophages, otherwise known as Kupffer cells. Secondly, comparison of PK2/Bv8 expression in isolated human primary hepatocytes, hepatic stellate cells, Kupffer cells and hepatoma cell lines, showed that only Kupffer cells express significant levels of PK2/Bv8. Immunostaining on normal liver serial sections of CD68 and PK2/Bv8 showed that all PK2/Bv8 positive cells were equally CD68

positive. Finally, colocalization of PK2/Bv8 with CD68, a Kupffer cell marker, carried out in an isolated Kupffer cell-enriched population showed that all cells positive for PK2/Bv8 were also CD68 positive. Taken all together these data validate the hypothesis that Kupffer cells are the specific source of PK2/Bv8 in liver. Interestingly, it is a well known event that the number of Kupffer cells decreases in cirrhosis and even more in HCC relative to normal liver[44]. In this study, we confirm this previous report and observe that the expression pattern of CD68, a marker for Kupffer cells, decreases in fibrosis and in HCC compared to normal liver. CD68 and PK2/Bv8 follow a similar down-regulation pattern, which is confirmed by a correlation between the two gene expressions. Thus, these results contribute towards a potential explanation that the down regulation of PK2/Bv8 in HCC is due to the gradual decrease in number of Kupffer cells during the progression of the pathology. However, based on our results we can not exclude a possible regulation of PK2/Bv8 in Kupffer cells by the hepatic micro-environment.

Our results showing that PK2/Bv8 producing cells are Kupffer cells; are consistent with the fact that PK2/Bv8 is expressed by several types of leukocytes including the monocyte/macrophage lineage[14,19]. Even though PK1/EG-VEGF was not expressed in Kupffer cells it has also been associated to leukocyte lineage. For instance, PK1/EG-VEGF expression was positively correlated to macrophages in corpus luteum regression and follicular atresia of bovine ovaries[45], and to tumor infiltrating lymphocytes of human ovarian carcinoma[29]. All together, PK1/EG-VEGF and PK2/Bv8 appear to be expressed by immune cells differentially according to the tissue. Furthermore, we characterized the cellular targets of PK2/Bv8 in liver by measuring the expression for PK2/Bv8's receptors, called PKR1 and PKR2, in the same panel of liver cells. Interestingly, PKR1 and PKR2 are most strongly expressed by Kupffer cells. Recent reports have demonstrated that PK2/Bv8 has potent cytokine properties, PK2/Bv8 has the ability to stimulate monocyte production, mobilization and differentiation into macrophage like cells[19,20]. Therefore we can speculate that, in normal liver macrophage physiology, PK2/Bv8 could act by an autocrine manner on the resident macrophage population in the liver sinusoids. PK2/Bv8 might regulate Kupffer cells function and be involved in monocyte recruitment and differentiation of Kupffer cells.

In conclusion, we demonstrate that PK2/Bv8 is expressed in resident Kupffer cells in normal liver and that the expression of PK2/Bv8 decreases significantly as the pathology evolves towards hepatocellular carcinoma.

ACKNOWLEDGMENTS

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COMMENTS

Background

Liver cancer is one of the most common types of cancer and is a solid tumor showing important angiogenesis. Angiogenesis is an essential step in tumor progression, where the creation of novel blood vessels allows the growth and dissemination of cancer cells. Disrupting cancer blood vessels by blocking the secreted peptides responsible for endothelial cell proliferation has become a novel axis of anti-cancer treatment. Thus, identifying new secreted molecules involved in liver cancer vascularization could provide novel therapeutic targets.

Research frontiers

The aim of this study was to evaluate if two novel secreted peptides PK1/EG-VEGF and PK2/Bv8, described as having potent angiogenic effects, were involved in the vascularization process of human hepatocellular carcinoma.

Related publications

The present study had for goal to evaluate the angiogenic potential of prokineticins in hepatocellular carcinoma. To this effect we cited several articles from other investigators reporting researches on prokineticin action as an angiogenic factor, their implication in several other types of cancer.

Innovations and breakthroughs

This study clearly shows that PK1/EG-VEGF and PK2/Bv8 are not involved in human hepatocellular carcinoma vascular progression. Furthermore, in liver PK2/Bv8, but not PK1/EG-VEGF, was detected and was specifically expressed only by liver resident macrophages, known as Kupffer cells. Moreover, Kupffer cells express high levels of prokineticin receptor 1 and 2 suggesting paracrine/autocrine regulation by PK2/Bv8.

Applications

Even though our results show that PK2/Bv8 is not associated with hepatocellular carcinoma, it brings novel data on Kupffer cell physiology. All together our findings, corroborate numerous other reports demonstrating that prokineticins are expressed by immune cells, and have potent immunomodulatory activities.

Terminology

Prokineticin 1 (PK1/EG-VEGF) and prokineticin 2 (PK2/Bv8) are small secreted peptides of 8 kDa that signal through two receptors that are members of the G-protein coupled receptors superfamily. In addition to its angiogenic activites, prokineticins have also been shown to be implicated in hematopoiesis, reproductive angiogenesis, neurogenesis, ingestive behaviour and hormone release, gastrointestinal motility, circadian rhythms, and pain sensation. Hepatocellular carcinoma (HCC) is the major type of liver cancer; it develops majoritarily on a cirrhotic background, and is highly angiogenic.

Peer review

This manuscript describes for the first time in detail the expression and localization of PK2/BV8 in human liver, and in general the manuscript is well written.

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BASIC RESEARCH

Probiotic *Leuconostoc mesenteroides* ssp. *cremoris* and *Streptococcus thermophilus* induce IL-12 and IFN-γ production

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Abstract

AIM: To investigate the capacity of potentially probiotic strains from six bacterial genera to induce cytokine production alone or in combinations in order to identify potential enhancing or synergistic effects in order to select probiotic bacteria for *in vivo* purposes.

METHODS: Cytokine production in human peripheral blood mononuclear cells (PBMC) in response to stimulation with eleven different potentially probiotic bacterial strains from *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Leuconostoc* and *Propionibacterium* genera was analysed. Production and mRNA expression of TNF-α, IL-12, IFN-γ and IL-10 were determined by ELISA and Northern blotting, respectively.

RESULTS: All tested bacteria induced TNF-α production. The best inducers of Th1 type cytokines IL-12 and IFN-γ were *Streptococcus* and *Leuconostoc* strains. All *Bifidobacterium* and *Propionibacterium* strains induced higher IL-10 production than other studied bacteria. Stimulation of PBMC with any bacterial combinations did not result in enhanced cytokine production suggesting that different bacteria whether gram-positive or gram-negative compete with each other during host cell interactions.

CONCLUSION: The probiotic *S. thermophilus* and *Leuconostoc* strains are more potent inducers of Th1 type cytokines IL-12 and IFN-γ than the probiotic *Lactobacillus* strains. Bacterial combinations did not result in enhanced cytokine production.

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Key words: Probiotics; *Streptococcus*; *Lactobacillus*; *Bifidobacterium*; *Lactococcus*; *Leuconostoc*; *Propionibacterium*; Immune response; Cytokines; Mononuclear cells

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INTRODUCTION

Probiotics are defined as living microorganisms that have beneficial effects on human health[1]. Documented health effects in human intervention trials include amelioration of acute diarrhoea in children, reduction of the risk of antibiotic-associated gastrointestinal symptoms, relief of milk allergy/atopic dermatitis in infants, reduction in the risk of atopic diseases and respiratory infections, relief of irritable bowel syndrome and rheumatoid arthritis symptoms, suppression of *H pylori* and modulation of the immune response[2-4]. The ways in which probiotic bacteria affect the human immune system *in vivo* and *in vitro* are not fully understood. The immunomodulatory effects of probiotic bacteria could be due to the produced cytokines that further regulate innate and adaptive immune responses.

Different leukocyte types cooperate during the activation of innate and adaptive immune responses. In addition to direct cellular contacts the communication between immune cells depends on secreted mediators including cytokines. Bacteria-induced stress produces inflammatory cytokines promoting the activation of antimicrobial immune responses. TNF-α is a potent

inducer of many inflammatory molecules including other cytokines[5]. IL-12 produced by activated antigen-presenting cells (APC) enhances the development of Th1 type immune responses and stimulates NK and T cell IFN-γ production. All this further enhances cell-mediated Th1 type responses[6]. IL-10 downregulates the inflammatory response and induces an antibody-mediated immune response[7]. In previous studies it has been shown that individual probiotic bacteria can induce the production of TNF-α[8-10], IL-12[11-15], IFN-γ[10,11,13,15] and IL-10[9,15-18] in human peripheral blood mononuclear cells (PBMC) *in vitro*. At present only a limited amount of comparative data is available on the ability of different probiotic strains to induce cytokine production within the same experimental system[9,14-17]. Also, the effect of probiotic bacterial combinations on cytokine production *in vitro* is not well documented.

Understanding of the cytokine patterns that probiotics elicit may help in designing probiotics for specific preventative or therapeutic purposes. Information on the cytokine production induced by different probiotic bacteria and their combinations would enable development and optimal clinical use of these microbes as health promoting substances. In the present study human PBMCs consisting of monocytes, T and B lymphocytes and NK cells, are used for *in vitro* screening for cytokine production. Cytokine production in response to stimulation with different potentially probiotic strains from six bacterial genera *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Leuconostoc* and *Propionibacterium* alone or in combinations is analysed in order to identify potential enhancing or synergistic effects.

MATERIALS AND METHODS

Bacterial strains

Streptococcus pyogenes serotype T1M1 (IH32030) and *Escherichia coli* (DH5α) were obtained from the collection of National Public Health Institute (Helsinki, Finland). Eleven potentially probiotic strains; *Streptococcus thermophilus* THS, *Lactobacillus rhamnosus* GG (ATCC 53103), *Lactobacillus rhamnosus* Lc705 (DSM 7061), *Lactobacillus helveticus* 1129, *Lactobacillus helveticus* Lb 161, *Bifidobacterium longum* 1/10, *Bifidobacterium animalis* ssp. *lactis* Bb12, *Bifidobacterium breve* Bb99 (DSM 13692), *Lactococcus lactis* ssp. *cremoris* ARH74 (DSM 18891), *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) and *Propionibacterium freudenreichii* ssp. *shermanii* JS (DSM 7067), were obtained from Valio Research Centre (Helsinki, Finland). Bacteria were stored in skimmed milk at -70℃ and passaged three times (except *Bifibobacterium* strains which were passaged four times) before they were used in stimulation experiments. *S. pyogenes* was grown at 37℃ under aerobic conditions in sheep blood agar (Oxoid, Ogdensburg, NY, USA) and tryptone-yeast broth supplemented with 2 g/L glucose[19], *E. coli* at 37℃ under aerobic conditions in Luria-medium (National Public Health Institute, Helsinki, Finland), *Lactobacillus rhamnosus* strains were grown at 37℃ under aerobic conditions in de Man, Rogosa and Sharpe (MRS) medium (Lab M, Topley House, Lancashire, UK), *Lactobacillus helveticus* strains were grown at 42℃ under aerobic conditions in MRS medium (Lab M, Topley House), *L. mesenteroides* was grown at 22℃ under aerobic conditions in MRS medium (Lab M, Topley House), *Bifidobacterium* strains at 37℃ under anaerobic conditions in MRS medium (Lab M, Topley House) with 5 g/L cysteine (Merck, Darmstadt, Germany), *S. thermophilus* at 37℃ under aerobic conditions in M17-agar (Lab M, Topley House) with 20 g/L lactose (J.T. Baker B.V., Deventer, Holland) and M17-broth (Difco, Beckton Dickinson, MD, USA) with 20 g/L lactose (J.T. Baker B.V.), *P. freudenreichii* at 30℃ under aerobic conditions in propioni-medium (Valio Ltd, Helsinki, Finland), *L. lactis* at 22℃ under aerobic conditions in calcium citrate agar (Valio Ltd) and M17-broth (Difco) with 20 g/L lactose (J.T. Baker B.V.). For stimulation experiments bacteria were grown to logarithmic growth phase, and the number of bacteria was determined by counting in a Petroff-Hauser counting chamber.

Cell culture

Human PBMC were purified by density gradient centrifugation over a Ficoll-Paque gradient (Amersham-Pharmacia Biotech, Uppsala, Sweden) from freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland)[20]. After washing, the cells were resuspended in RPMI 1640 medium (Sigma, St. Louis, Mo., USA) containing 10% heat-inactivated fetal calf serum (Integro, Zaandam, Holland) and supplemented with 2 mmol/L L-glutamine (Sigma), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco BRL, Paisley, Scotland). In stimulation experiments purified leukocytes (2×10^9 cells/mL) were incubated with bacteria in a final volume of 1 mL in 24-well plates (Nunc, Roskilde, Denmark) in 5% CO_2 at 37℃.

Stimulation experiments

All experiments were performed with cells obtained from four different blood donors. During bacterial stimulations PBMCs were maintained in RPMI-1640 medium containing 100 mL/L FCS. Bacteria were added into the cell culture to obtain the required bacteria: host cell ratio. *S. pyogenes* was used as a positive control and RPMI 1640 containing 100 mL/L FCS as a negative control. Bacterial doses and incubation times are as indicated for each experiment. When PBMC were stimulated with a combination of two bacteria or more, equal numbers of different bacteria were used, and the sum bacterial dose of the combinations was 10:1 of a bacteria: host cell ratio. Cell culture supernatants were collected from individual donor cell cultures and stored at -20℃ before analysis. For RNA analysis cells from different donors were pooled.

Cytokine specific ELISA

Cell culture supernatants obtained from individual donors were analyzed for cytokine levels by using enzyme-linked immunosorbent assay (ELISA) essentially as previously described[8]. TNF-α and IL-10 were determined with antibody pairs and standards obtained from BD Pharmingen (San Diego, CA, USA). IFN-γ and IL-12p70 were determined with Eli-pair kits (BioSite, Täby, Sweden).

RNA isolation and Northern blotting

For isolation of total cellular RNA, stimulated cells from different donors were pooled, collected, washed with PBS, and lysed in guanidinium isothiocyanate[21], followed by a centrifugation through a CsCl cushion as previously described[22]. RNA was quantified photometrically and samples containing equal amounts (10 μg) of total cellular RNA were size-fractioned on 1% formaldehyde-agarose gels, transferred to Hybond-N nylon membranes (Amersham-Pharmacia-Biotech) and hybridized. To control equal loading, ethidium bromide staining was used. The cDNA probes were human TNF-α (ATCC), IL-12 p40 and p35[23], IFN-γ[24] and IL-10 (DNAX, Palo Alto, USA). Hybridizations were performed in a solution containing 500 g/L formamide, 5 × Denhardt's solution, 5 × SSPE and 5 g/L SDS at 42℃. After hybridization membranes were washed three times with 1 × saline sodium citrate/g per L SDS at 42℃ for 30 min and once at 65℃ for 30 min. Membranes were exposed to Kodak X-Omat AR films (Eastman Kodak, Rochester, NY, USA) at -70℃ with intensifying screens.

RESULTS

Bacterial dose-dependent induction of cytokine production in human PBMC

To determine the optimal bacterial dose that can induce cytokine production in human PBMC, cells were stimulated with different doses of live probiotic bacteria (2:1, 10:1 and 50:1 bacteria: cell ratio). Cell culture supernatants were collected at 24 h after stimulation and cytokine levels were determined by ELISA. As shown in Figure 1 all bacteria induced TNF-α production and *L. mesenteroides* was the most potent inducer. IL-12 and IFN-γ were best induced by streptococci and *L. mesenteroides*. Bifidobacteria induced IL-10 production in a dose-dependent manner, while other probiotic species were weak inducers of IL-10 production (Figure 1). Bacteria: host cell ratio of 10:1 was chosen for more detailed analyses.

Kinetics of cytokine production in probiotic bacteria-stimulated PBMC

Next the kinetics of cytokine production in PBMC using a 10:1 bacterium: host cell ratio was analysed by ELISA. All probiotic bacteria induced TNF-α production by 6 h after stimulation (Figure 2). With *S. thermophilus*, *Lactobacillus* strains, *B. longum* and *B. breve* maximal TNF-α production levels were reached at 12 h after stimulation (Figure 2). Other measured cytokines were secreted later, and all tested probiotic bacteria induced maximal IL-12, IFN-γ and IL-10 production at 24 h after stimulation (Figure 2). There were considerable differences in the ability of studied bacteria to induce cytokine production in PBMC. *S. thermophilus* and *L. mesenteroides* were the best inducers of proinflammatory cytokines TNF-α, IL-12 and IFN-γ. Anti-inflammatory IL-10 production was induced by all *Bifidobacterium* strains and *P. freudenreichii*. All strains of *Lactobacillus* were weak inducers of IL-10 and Th1 type cytokines.

Probiotic bacteria-induced cytokine mRNA expression

Those bacteria that were the best inducers of cytokines at the protein level (namely *S. thermophilus*, *B. breve*, *L. mesenteroides* and *P. freudenreichii*) were selected for more detailed analysis of cytokine mRNA expression as analyzed by Northern blotting. In addition to our positive control *S. pyogenes*, also *L. rhamnosus* GG that has been extensively studied *in vitro* by our group and in many clinical trials[2-3] was added for the analysis. Consistent with the kinetics of TNF-α protein production, mRNA expression was fast and detectable at 3 h after bacterial stimulation (Figure 3). With all studied bacteria, the kinetics of TNF-α mRNA expression was similar. IL-12 p40, IFN-γ and IL-10 genes were induced later than TNF-α and their expression was detectable starting at 9 h after stimulation (Figure 3). *S. thermophilus*, *B. breve* and *L. mesenteroides* induced IL-12 p40 mRNA expression as well as *S. pyogenes*, a well-known inducer of IL-12 p40 in PBMCs (Figure 3)[11]. *S. thermophilus* and *L. mesenteroides* induced IL-12 p40 mRNA expression at 9 h while *P. freudenreichii* induced the expression only weakly. IL-12 p35 mRNA expression remained under the detection limit (data not shown). The kinetics of IFN-γ mRNA expression differed from that of IL-12 p40. In probiotic bacteria-stimulated PBMCs IFN-γ mRNA expression was observed at 9 h after stimulation, and its expression increased until the 24 h time point. *L. mesenteroides*, *S. thermophilus* and *B. breve*, in this rank order were able to induce IFN-γ mRNA expression (Figure 3). IL-10 mRNA expression was highest at 9 h after bacterial stimulation persisting until the 24 h time point. Interestingly, *B. breve* and *P. freudenreichii* were able to induce IL-10 mRNA expression already at 3 h after stimulation (Figure 3). The kinetics of probiotic bacteria-induced cytokine mRNA expression was in line with the cytokine secretion (Figures 2 and 3).

Combination effect of probiotic bacteria on cytokine production in human PBMC

The probiotic bacteria and their combinations which have been used in clinical trials, namely *L. rhamnosus* strains, *P. freudenreichii* and *B. breve*[25-28], *L. rhamnosus* strains, *P. freudenreichii*, and *B. animalis*[29,30] and *L. rhamnosus* strains and *P. freudenreichii*[31], were used to study whether the effect of probiotic combinations differs from an individual strain. All probiotic combinations induced TNF-α production at the same or lower level as compared to those responses induced by probiotic bacteria alone (Figure 4). IL-12 and IFN-γ production induced by all probiotic bacterial combinations was weak (Figure 4). Also the IL-10 production by the probiotic combinations was lower than by individual *Bifidobacterium* strains (Figure 4) In general; the responses induced by the combinations of probiotic bacteria were an average of responses induced by each bacterium alone (Figure 4). In addition the representatives of the best cytokine inducers in this study, namely *S. thermophilus*, *Bifidobacterium* strains, *L. mesenteroides* and *P. freudenreichii* and our controls *S. pyogenes* and *L. rhamnosus* GG were analysed for their possible additive and synergistic effects on cytokine production in PBMCs. Two different probiotic bacteria were used simultaneously

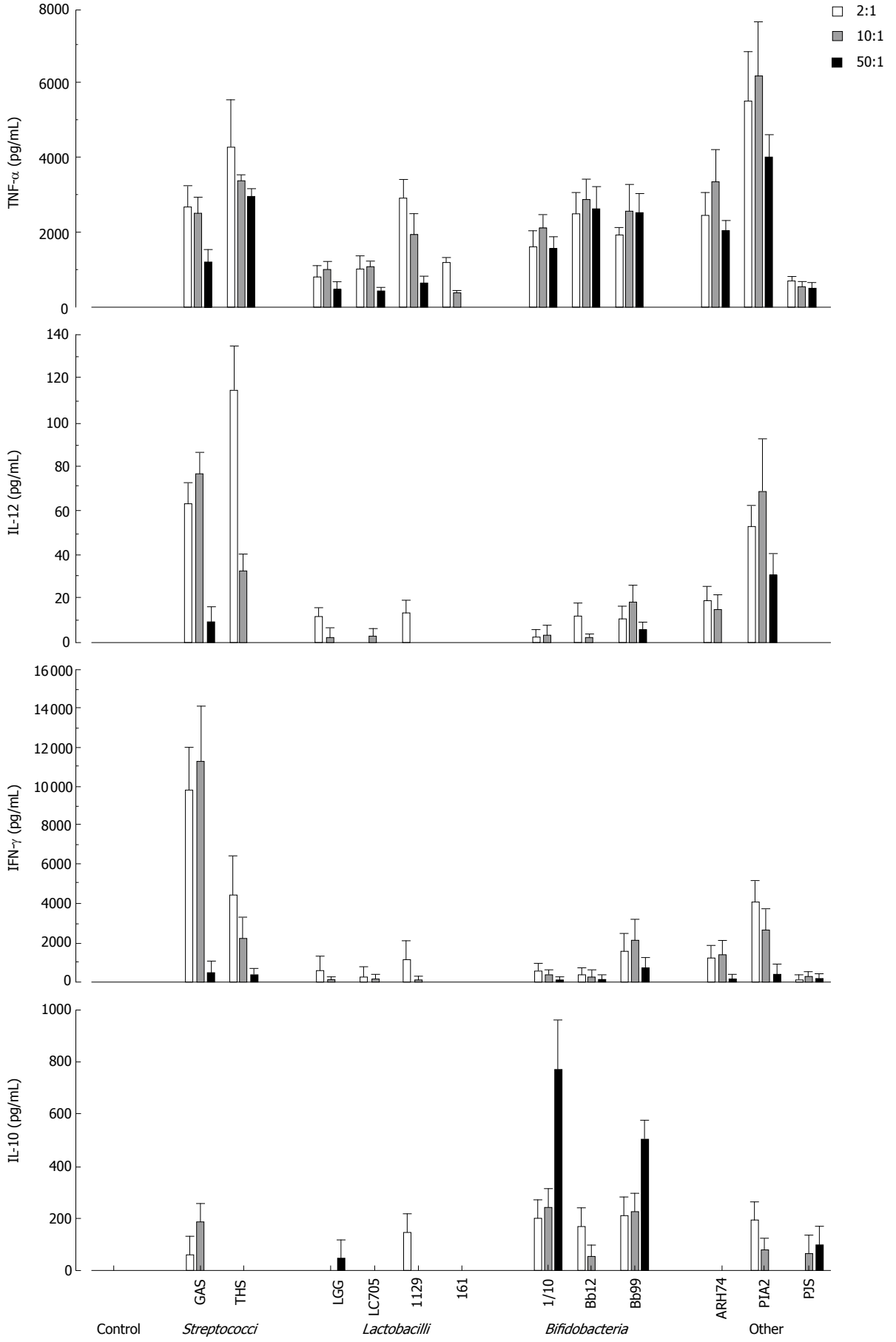


Figure 1 Dose-dependent cytokine production in human PBMC (2×10^6/mL) stimulated with *Streptococcus pyogenes* and 11 probiotic bacteria (2, 10 and 50 bacteria/cell) for 24 h, mean ± SEM, $n = 4$. A representative experiment out of two is shown. GAS: *Streptococcus pyogenes*; THS: *Streptococcus thermophilus* THS; LGG: *Lactobacillus rhamnosus* GG; LC705, *Lactobacillus rhamnosus* LC705; 1129: *Lactobacillus helveticus* 1129; 161: *Lactobacillus helveticus* Lb 161; 1/10: *Bifidobacterium longum* 1/10; Bb12: *Bifidobacterium animalis* ssp. lactis Bb12; Bb99, *Bifidobacterium breve* Bb99; ARH74: *Lactococcus lactis* ssp. *cremoris* ARH74; PIA2, *Leuconostoc mesenteroides* ssp. *cremoris* PIA2; PJS: *Propionibacterium freudenreichii* ssp. *shermanii* JS.

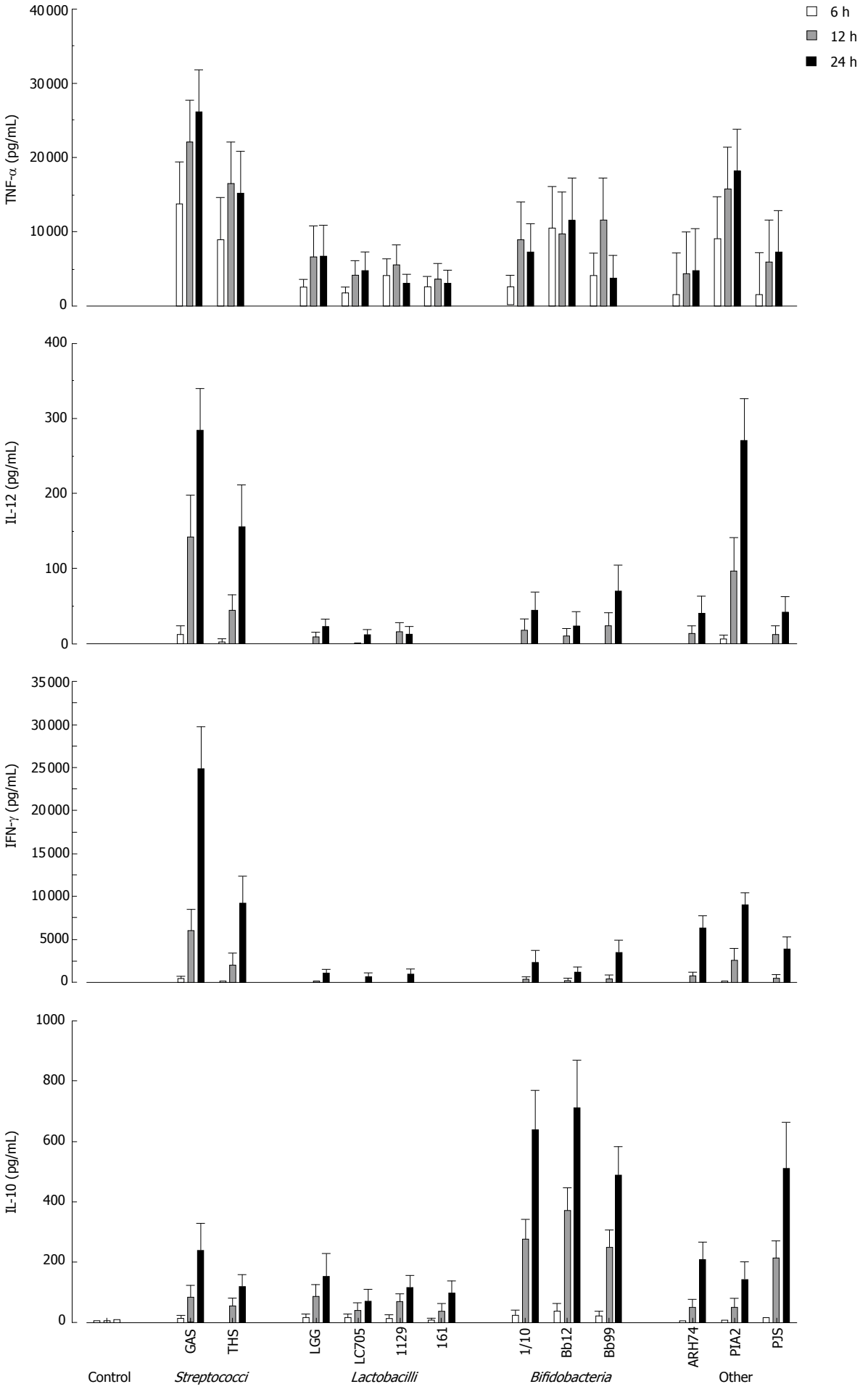


Figure 2 Kinetics of cytokine production in probiotic bacteria-induced human PBMC. (10 bacteria/cell, mean ± SEM, *n* = 4). See Figure 1 for abbreviations of bacteria.

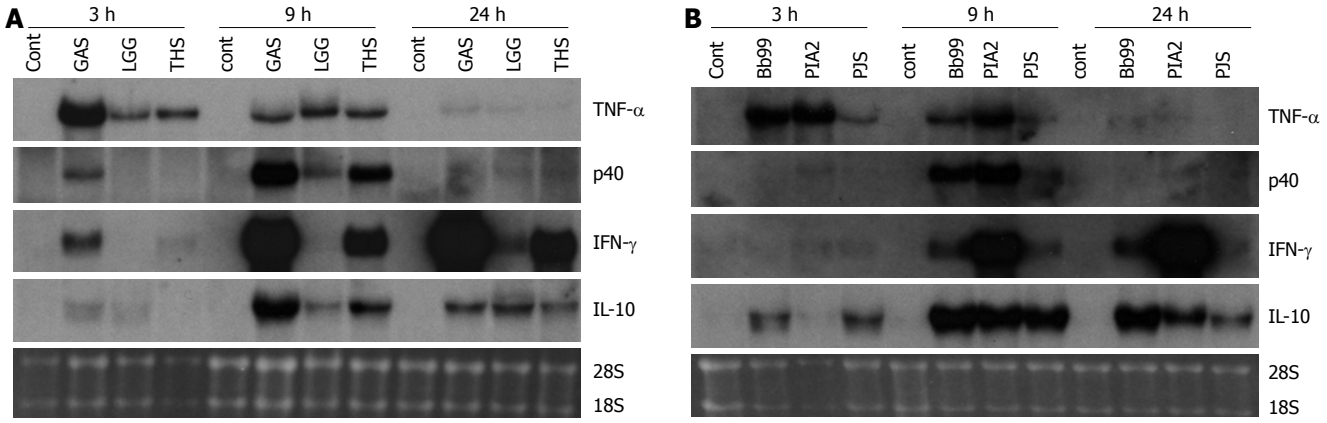


Figure 3 Kinetics of cytokine mRNA expression in probiotic bacteria-induced human PBMC (pooled samples from four donors), 10 bacteria/cell, 10 μg RNA/lane on 10 g/L agarose gel and Northern blot analysis with indicated cDNA probes was carried out. Ethidium bromide staining of gels was used to verify equal loading of RNA. See Figure 1 for abbreviations of bacteria. **A**: *S. pyogenes*, *L. rhamnosus* GG or *S. thermophilus* and **B**: *B. breve*, *L. mesenteroides*, *P. freudenreichii*.

to stimulate PBMCs for 24 h with a bacteria: host cell ratio of 10:1 and cell culture supernatants were collected for ELISA analysis. None of these probiotic bacterial combinations induced additive or synergistic cytokine production in PBMCs. On the contrary, the responses induced by the combinations were closer to an average of responses induced by the individual bacteria (Figure 5).

Effect of probiotic bacteria together with E. coli on cytokine production in human PBMC

All studied probiotic bacteria are gram-positive and thus likely to use Toll-like receptor (TLR)2-mediated signal transduction pathways in host cells while a gram-negative bacterium *E. coli* uses TLR4[32]. We did not observe any additive effects on cytokine production induced by the combinations of our gram-positive probiotic bacteria (Figure 5). Since this could be due to our probiotic bacteria engaging the same receptor, we wanted to analyse whether individual probiotic bacteria combined with *E. coli* would have an effect on cytokine production. Probiotic *S. thermophilus*, *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Leuconostoc* or *Propionibacterium* strain and *E. coli* were combined and cell culture supernatants were collected at 24 h after bacterial stimulation followed by determination of cytokine production by ELISA. *E. coli* induced TNF-α, IFN-γ and IL-10 production, but not that of IL-12 (Figure 6). Stimulation of PBMCs with different combinations of probiotic bacteria together with *E. coli* lead to the induction of TNF-α production, but the response was an average of responses with individual bacteria (Figure 6). *E. coli*-induced IFN-γ production was reduced when it was combined with *Lactobacillus* strains, *P. freudenreichii* and *B. longum* (Figure 6). No enhancement of IL-10 production was seen in cells stimulated with the combinations of *E. coli* and probiotic bacteria.

DISCUSSION

In this study we have systematically analysed the ability of potentially probiotic bacterial strains from six different genera; *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Leuconostoc* and *Propionibacterium*, to induce cytokine expression *in vitro* in human PBMC. We show that eleven probiotic strains induce cytokine expression differently and the expression pattern seems to be dependent on the bacterial genera. In addition, stimulation of PBMC with any bacterial combinations, whether gram-positive or gram-negative, does not lead to enhanced cytokine production. Also we report for the first time that novel probiotic *S. thermophilus* and *Leuconostoc* strains are more potent inducers of Th1 type cytokines IL-12 and IFN-γ than the probiotic *Lactobacillus* strains presently in clinical use.

Human PBMC offer a model for studying the potential of different probiotic strains to induce cytokine production. *In vivo*, probiotics are not in direct contact with PBMC. Instead, probiotics interact with the epithelial cells of the gut, and probiotics may be taken up by macrophages, dendritic cells or M-cells at Peyer's patches and this can lead to the activation of lymphocytes such as monocytes residing in Peyer's patches[33]. Since PBMC are a source for monocytes among other immune cells, they provide an adequate model to study the immunological properties of probiotic bacteria. Therefore, *in vitro* bacteria-host cell studies aid in selecting novel probiotic strains for clinical trials.

Our data is consistent with previous studies showing that probiotic bacteria are able to induce TNF-α secretion in human PBMC[8,10,11,17]. However, there were considerable differences in the ability of different probiotic bacteria to induce IL-12 and IFN-γ. Interestingly, *Lactobacillus* and *Bifidobacterium* strains which have previously been shown to stimulate IL-12 and IFN-γ production in human PBMC[11-14,17], were in the present study found to be relatively poor inducers of these cytokines. Instead, we found that novel probiotic *S. thermophilus* and *Leuconostoc* strains were extremely good inducers of these Th1 type cytokines. One of the approaches in treating allergy could be tipping the Th1/Th2 balance from Th2 predominance to Th1 type response. In clinical trials *L. rhamnosus* GG[27,34,35] or a combination of two different *L. rhamnosus* strains, *Bifidobacterium* and *Propionibacterium*[28] have been used successfully to prevent atopic diseases. A strong Th1 type cytokine response is also an important factor

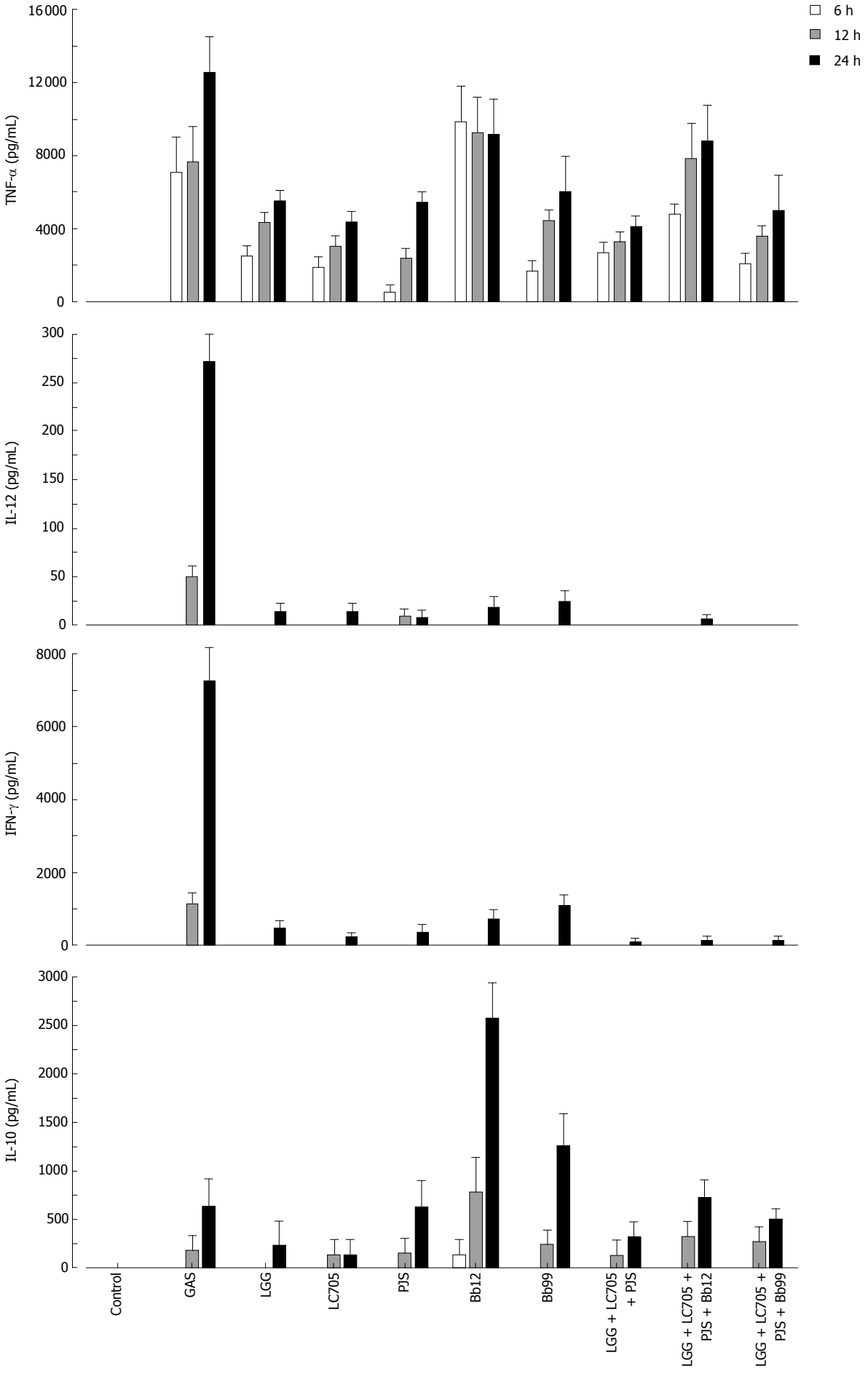


Figure 4 Kinetics of cytokine production in human PBMC stimulated with bacterial combinations. (10 single bacteria/cell, or three bacteria/cell in the combinations, *n* = 4). See Figure 1 for abbreviations of bacteria.

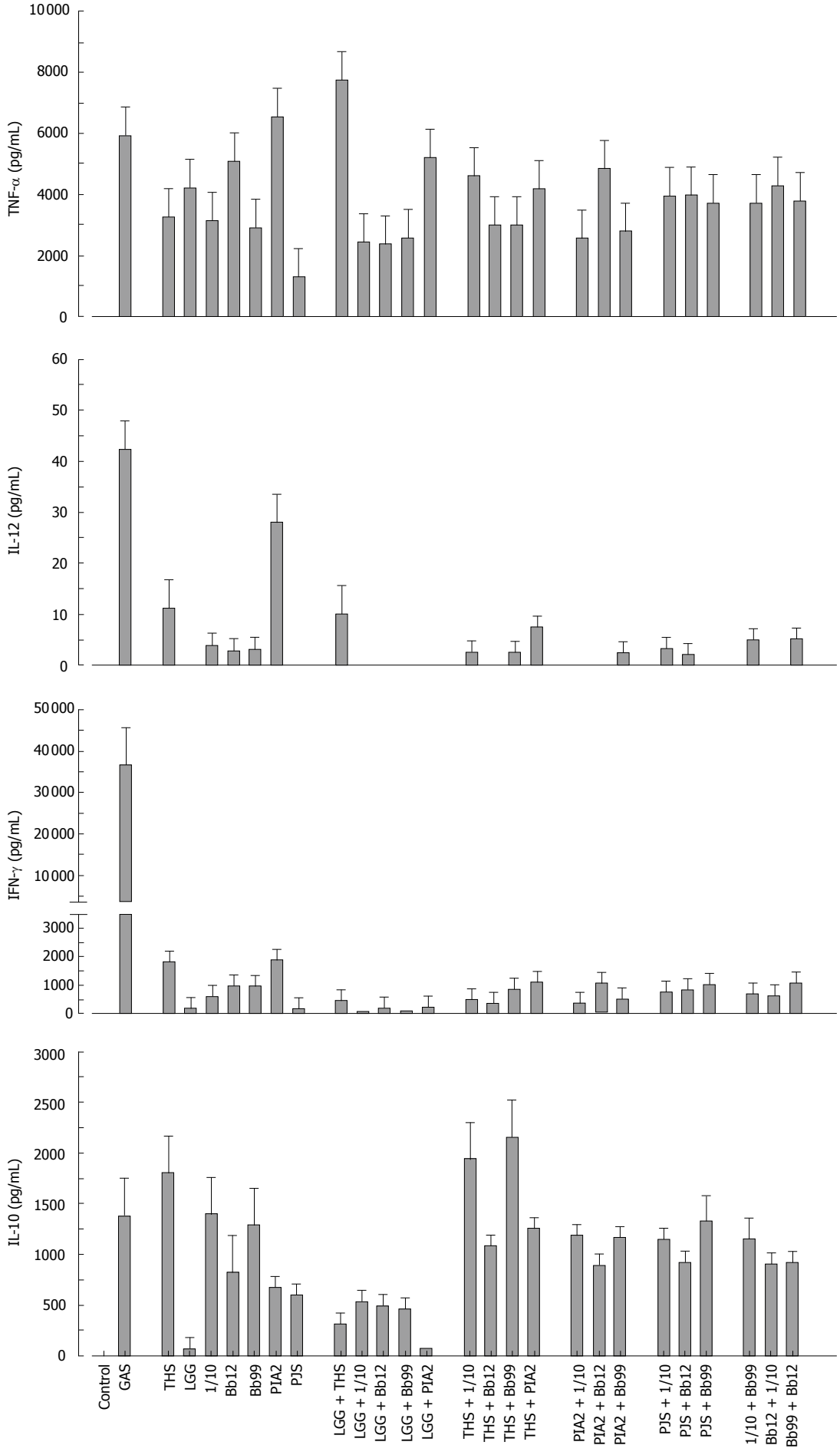


Figure 5 Cytokine production in human PBMC stimulated with probiotic bacterial combinations. (10 single bacteria/cell or their combinations at 5/cell for 24 h, mean ± SEM, *n* = 4). See Figure 1 for abbreviations of bacteria.

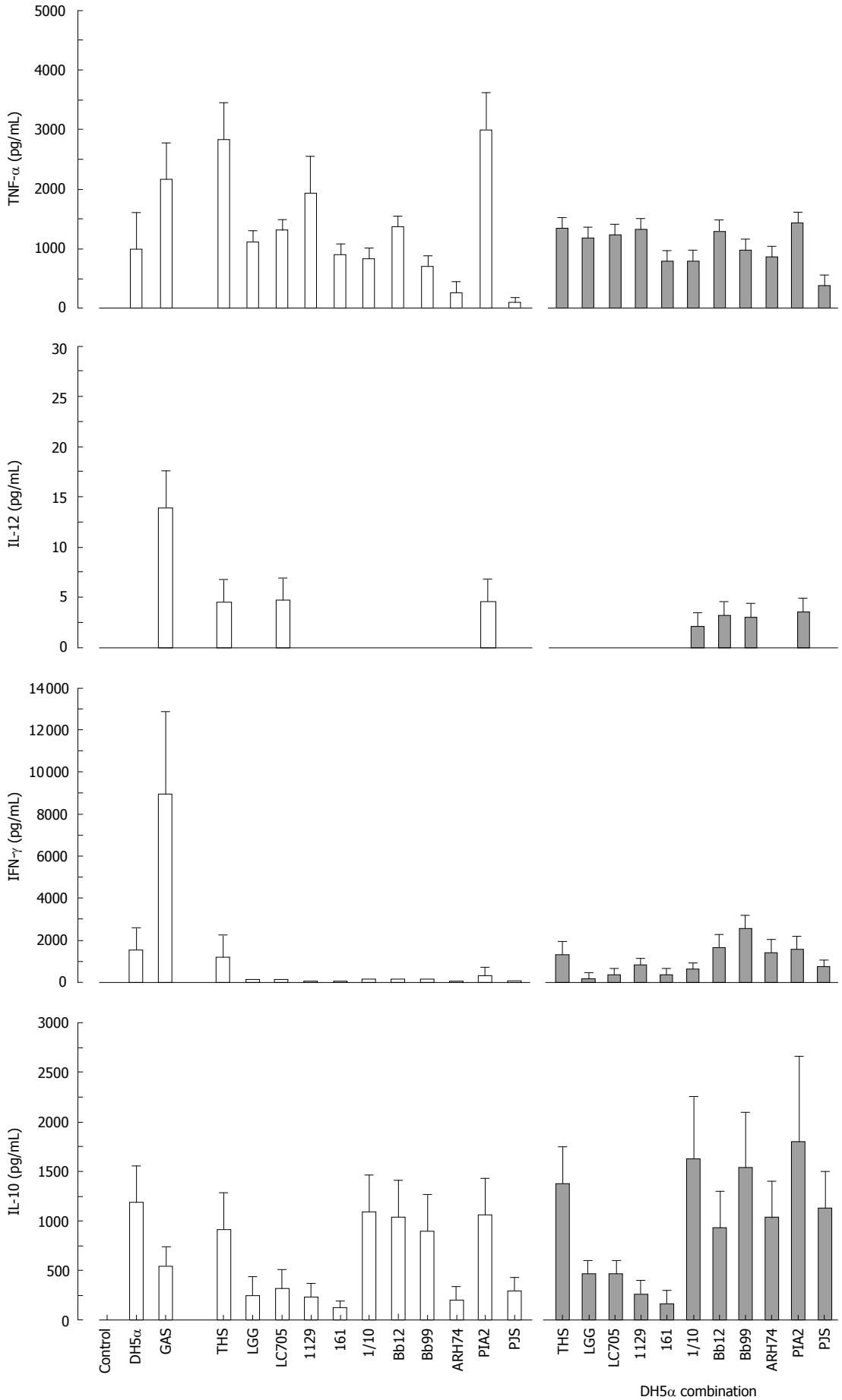


Figure 6 Stimulation of PBMC with a combination of gram-positive probiotic bacteria and a gram-negative bacteria for 24 h. (*E. coli* DH5α or different probiotic bacteria alone at 10 bacteria/cell, or *E. coli* combined with each probiotic bacterium at 5 bacteria/cells means ± SEM, $n = 4$). Abbreviations of bacteria see Figure 1.

in the fight against viral infections such as that caused by influenza A virus. Indeed, *Lactobacillus* strains have been shown to prevent and ameliorate the symptoms of respiratory infections[36-40]. It is of interest that for example *L. rhamnosus* GG has been effective in immune mediated diseases even though in the present study we observed that LGG induced very low cytokine production. This may be due to the fact that cytokine induction is only one of the proposed mechanisms of action for probiotics. Other as important factors may for example be the ability to adhere to the gut epithelium and the promotion of non-immunological gut defence barrier by normalizing permeability and disturbed gut microecology. Since *S. thermophilus* and *Leuconostoc* strains used in the present study were extremely potent inducers of IL-12 and IFN-γ, these strains may show better clinical efficiency in enhancing Th1 response in allergy and in the prevention of respiratory infections than the presently used probiotic *Lactobacillus* strains.

IL-10 was induced by *Bifidobacterium* and *Propionibacterium* strains, whereas IL-10 production induced by *Streptococcus*, *Lactobacillus*, *Lactococcus* and *Leuconostoc* strains remained at a low level. Our data is consistent with previous studies in which bifidobacteria were shown to induce higher IL-10 production as compared to lactobacilli[9,16]. The anti-inflammatory actions of IL-10 could be helpful in the treatment of inflammatory conditions or diseases. There is preliminary evidence that probiotics could be used in the treatment of inflammatory diseases like ulcerative colitis, pouchitis and rheumatoid arthritis[2,41]. The amelioration of these inflammatory diseases could be due to the induction of IL-10. Recent studies indicate a possible role of low-grade mucosal inflammation also in the pathogenesis of irritable bowel syndrome[42,43]. Preliminary evidence exists that a combination of probiotics, which included anti-inflammatory *Bifidobacterium* and *Propionibacterium* strains in addition to two different *L. rhamnosus* strains, relieves the symptoms of irritable bowel syndrome[25]. *Bifidobacterium* and *Propionibacterium* which in this study were able to induce anti-inflammatory IL-10 production could thus be used to treat different types of inflammatory diseases.

The use of probiotic bacterial combinations in clinical trials has shown great promise making it important to understand the immunological properties of a single strain versus different bacterial combinations. However, there is only limited amount of comparative data on the immunomodulatory properties of several different probiotic bacterial strains within the same experimental system. Published data is mainly limited to the analysis of the effects of *Lactobacillus* and *Bifidobacterium* genera[9,11-13,44]. In the present study combinations of different gram-positive probiotic bacteria did not induce any additive or synergistic cytokine production in PBMCs. This could be due to the fact that all gram-positive bacteria are likely to use the same or similar intracellular signal transduction mechanisms to induce cytokine gene expression. Interestingly, no additive or synergistic induction of cytokine production was seen even when gram-positive probiotic bacterial strains were combined with a gram-negative bacteria, *E. coli*. On the contrary, *E. coli*-induced IFN-γ production was reduced when different probiotics were present during the stimulation experiments. Gram-positive and gram-negative bacteria have been shown to induce quite different cytokine production patterns.

In human PBMCs gram-positive bacteria induce TNF-α[8-10], IL-12[11-15] and IFN-γ[10,11,13,15] while gram-negative bacteria have preferentially been suggested to induce IL-10 production[14]. Gram-positive bacteria or their structural components activate cells *via* TLR2, whereas gram-negative bacteria and their major structural component lipopolysaccharide activate host cell *via* TLR4[32]. It is, however, likely that other receptor systems apart from TLRs take part in host cell responses to different microbes[45]. Our data suggests that different bacteria whether they are gram-positive or gram-negative compete with each other during bacteria-host cell interactions. Therefore, combining gram-positive and gram-negative bacteria to activate the respective TLR2 and TLR4-dependent signalling pathways does not further enhance human PBMC responses. Initial analyses with TLR2 and TLR4 ligand combinations also fail to demonstrate any synergistic enhancement in cytokine production (data not shown).

Our results suggest that probiotic bacteria in a genera-specific way direct immune responses to either the Th1 type or the anti-inflammatory side. Besides providing possible explanations for phenomena observed in clinical trials, this finding might enable pinning down the probiotic bacterial (genera) specific factors contributing to the type of immune response elicited. Understanding of the immunological properties of probiotic bacteria is needed in the development of probiotic bacteria for targeted treatment of different disease conditions.

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COMMENTS

Background and research frontiers

Probiotic bacteria have been used for the prevention and treatment of a diverse range of disorders. However, the ways in which probiotic bacteria elicit their health effects are not fully understood. One of the action mechanisms could be the ability to induce cytokines that further regulate innate and adaptive immune responses.

Innovations and breakthroughs

At present there is only a limited amount of comparative data available on the ability of different probiotic strains to induce cytokine responses within the same experimental system. Also, the effect of probiotic bacterial combinations on cytokine production *in vitro* is not well documented although bacterial combinations have been used in many clinical trials. In the present study we have analysed the cytokine production of eleven different potentially probiotic strains from six bacterial genera alone or in combinations in human peripheral blood mononuclear cells in order to indentify potential enhancing or synergistic effects.

Applications

We found that probiotic bacteria direct immune responses to either Th1 type or anti-inflammatory way in a bacterial genera-specific manner. The probiotic *S. thermophilus* and *Leuconostoc* strains are more potent inducers of Th1 type

cytokines IL-12 and IFN-γ than the probiotic *Lactobacillus* strains. Bacterial combinations did not result in enhanced cytokine production. More detailed information on the cytokine patterns that probiotic bacteria elicit may help in designing probiotics for specific preventative or therapeutic purposes.

Terminology

Probiotic bacteria are defined as living microorganisms that have beneficial effects on human health.

Peer review

This paper explores a mechanism of action of probiotics, namely the induction of cytokines by different strains of probiotics. The authors have designed a clear, logical study of different strains, dose-response, time of response and interactions of different probiotic strains. The paper includes an excellent exploration of probiotic mixtures.

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BASIC RESEARCH

Establishment and characterization of a new cell line derived from human colorectal laterally spreading tumor

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Abstract

AIM: To study the molecular mechanism of laterally spreading tumor (LST), a cell line [Laterally Spreading Tumor-Rectum 1 (LST-R1)] was derived and the characteristics of this cell line were investigated.

METHODS: A new cell line (LST-R1) originated from laterally spreading tumor was established. Properties of the cell line were characterized using scanning and transmission electron microscopy, immunohistochemistry method, cytogenetic analysis and nude mice xenograft experiments. *In vitro* invasion assay, cDNA microarray and Western blotting were used to compare the difference between the LST-R1 and other colorectal cancer cell lines derived from prudent colon cancer.

RESULTS: Our study demonstrated that both epithelial special antigen (ESA) and cytokeratin-20 (CK20) were expressed in LST-R1. The cells presented microvilli and tight junction with large nuclei. The karyotypic analysis showed hyperdiploid features with structural chromosome aberrations. The *in vivo* tumorigenicity was also demonstrated in nude mice xenograft experiments. The invasion assay suggested this cell line has a higher invasive ability. cDNA microarray and Western blotting show the loss of the expression of E-cadherin in LST-R1 cells.

CONCLUSION: We established and characterized a colorectal cancer cell line, LST-R1 and LST-R1 has an obvious malignant tendency, which maybe partially attributed to the changes of the expression of some adhesion molecules, such as E-cadherin. It is also a versatile tool for exploring the original and progressive mechanisms of laterally spreading tumor and the early colon cancer genesis.

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Key words: LST-R1; Cell line; Invasion; E-cadherin

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INTRODUCTION

Two routes have been described that can lead to colon carcinogenesis: one is the adenoma-carcinoma model, which is the main carcinogenetic pathway of prudent colorectal tumor and has been widely accepted in the west. However, an increasing number of superficial or flat colorectal tumors have been reported, particular in Japan[1,2]. The superficial tumors are considered to have a distinct clinicopathologic, genetic feature and behave a different carcinogenetic pathway, called de novo pathway[3-6]. Laterally spreading tumor (LST) is a unique subtype of superficial colorectal tumor, which was first reported by Kudo S in 1993[7,8] and many names such as granular cluster type, nodule-aggregating tumor, creeping tumor, superficial spreading (epithelial) tumor, flower-bed-like lesion had been used. In 1998, it was defined as tumors originated from the colorectal mucous membranes and mainly extends laterally rather than vertically with its diameter greater than 1 cm (Figure 1A)[9-12]. It has a tendency to affect the rectum, sigmoid colon, and caecum. In some studies, it has been reported that the carcinogenesis rate of LST is 8.64%-52.5%[13-15].

Moreover, our previous study has also shown that the incidence of LST is not low in China[16]. Flat appearance makes it difficult to detect it and previous study has shown that laterally spreading tumors have a higher malignant potential than conventional adenomas[2,17,18]. To study the distinctive biological behavior and the underlying biological mechanisms responsible for LST cancer progression will certainly help improving the detection rate of LST and eventually lead to the decreasing morbidity of colon cancer.

Invasion is an important step in the intricate process leading to the formation of cancer and has close relationship with the adhesion ability of the cancer cells. To investigate the higher malignant tendency of colorectal superficial tumor, we established a stable cell line derived from LST and assessed its invasion ability by *in vitro* invasion assay. Here we show that the invasion ability of LST is higher than SW480 and lovo cells, which is originated from prudent colon cancer. To elucidate the reason for this difference, we have developed a cDNA microarray, representing 18000 cDNA clusters to profile the gene expression patterns in Laterally Spreading Tumor-Rectum 1 (LST-R1), SW480, lovo cell lines and found that many genes associated with adhesion showed a different expression profile. Our data suggest that LST-R1cells have some distinct characteristics comparing with SW480 and lovo cells. Further investigations on the cells should enhance our understanding on the distinct biology of LST.

MATERIALS AND METHODS

Tumor origin

Laterally spreading tumor cells were derived from a rectal LST of 59-year-old Chinese woman. Magnifying endoscope showed a flat granular tumor with nodus (about 70 mm × 60 mm) in rectum, 3 cm far from anus. Examination of the biopsy specimen revealed that it has the characteristic morphology of a villous adenoma accompanied by moderate sever atypical hyperplasia (Figure 1B).

Tissue and cell culture

Tissue specimens were obtained by endoscopic partial mucosal resection (EPMR), and were transferred to a transport medium containing five folds penicillin, streptomycin (Invitrogen, Carlsbad, CA) and amphotercin B. The specimens were vibrated for about ten minutes to get rid of filth and washed five times with transport medium. They were then trimmed to remove fat and connective tissue, minced into pieces in a sterile culture dish, and subsequently plated in a 25 cm^2 flask. Cells were incubated in a 37℃ incubator with 5% CO_2. RPMI1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA)was added to the cells four hours later and media was changed every 2-3 d. Clonal growth was observed after 27 d of culture, and colonies were identified and subcultured. After 2 passages, the cells grew rapidly and were polymorphic with a few fibroblasts. To purify the cells, they were inoculated in 96-well plates and were cultured separately to get rid of fibroblasts. Finally, the polymorphic cells (more than 90%) were designated as LST-R1.

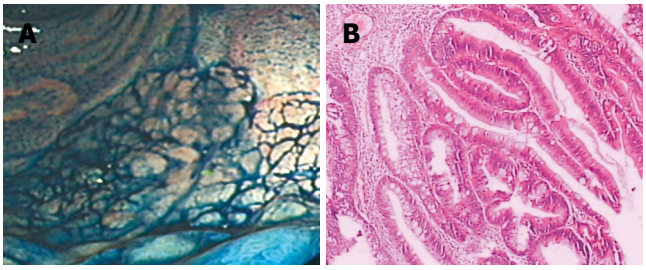


Figure 1 Endoscope and the histopathology of LST. **A**: LST under endoscope which grows laterally in the rectum, 3 cm far from the anus and has a diameter of 60 mm × 70 mm; **B**: The histopathology of the tissue: a villous adenoma accompanied by moderate severe atypical hyperplasia.

HCT116, lovo, SW480 and colo205 colon tumor cell lines were commercially obtained from the American Type Culture Collection and maintained in RPMI 1640 medium supplemented with 100 U/mL penicillin,100 μg/mL streptomycin, 10% fetal bovine serum in 75 cm^2 tissue culture flasks at 37℃ in a 5% CO_2 environment.

Confirmation of epithelial origin

Cells were grown on chamber slides and stained for CK20 and ESA expression with a specific antibody (Beijing ZhongShan Biology technology Ltd, Beijing, China).

Scanning and transmission electron microscopy

LST cells grown on slides were trypsinized and centrifuged. After fixed in 2.5% glutaraldehyde and postfixed in 1% osmium, they were dehydrated with acetone. The sample was divided into two parts: one half was added with acetas, dried on critical point of CO_2 and observed under scanning electron microscope, another half was embedded in paraffin and cut into thin pieces with a thickness of about 700 mm, stained with uranyl acetate and citrate, and observed under transmission electron microscope.

Metaphase chromosome preparation and spectral karyotyping (SKY)

Metaphase cells were obtained by treatment of the cultured cells with Colcemid (Gibco, Grand Island, NY, USA) at a final concentration of 0.03 μg/mL for 3 h. The harvested cells were treated with 0.8% sodium citrate for 15 min at 37℃ and fixed in 3:1 methanol/acetic acid. Metaphase chromosome spreading was performed as previously described[19]. The slide with metaphase cells was aged at room temperature (R.T.) for 5-7 s prior to SKY. The slide was treated with DNase-free RNase solution (0.1 g/L) at 37℃ for 1 h, and briefly rinsed in distilled water. After washing in 2 × SSC for 10 min at R.T, the slide was treated with proteinase K (0.5 μg/mL) solution for 10 min at 37℃ and washed in 2 × SSC at R.T. for 10 min. The slide was then fixed in 1% paraformaldehyde and washed in 2 × SSC at R.T. for 10 min each, followed by dehydration in 70%, 85%, and 95% ethanol at R.T. for 2 min. The slide was placed in 70% formamide/2 × SSC at 70℃ for 4 min and dehydrated in 70%, 85%, and 95% ethanol for 2 min

each. Other protocols including SKY probe (Applied Spectral Imaging, Migdal Ha'Emek, Israel) denaturation, hybridization and detection were done according to the recommendations of SKY probe manufacturer. SKY image capturing and karyotyping were performed using the SkyVision Imaging System equipped with a Zeiss Axioplan 2 fluorescence microscope. Recommendations were followed in karyotyping descriptions[20].

In vitro and in vivo growth properties of the cells

To determine the population doubling time and mitotic index of LST-R1 cells, the cells were suspended at a concentration of 1×10^5 per mL and 100 μL suspension was cultured in each well of 96 well plates. The cell growth was monitored after 12 h, 24 h, 36 h and 48 h by 3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT, the final concentration = 0.5 g/L) assay. The value indicated the mean of three independent experiments.

In vivo tumorigenicity studies were performed using 5-wk-old athymic nude mice obtained from the Animal Center of the First Military Medical University (Guangzhou, China). LST-R1 cells at passage 51 were trypsinized, washed twice with serum-free RPIM1640 medium, and resuspended in serum-free RPMI-1640 (1×10^6 per mL). A final volume of 0.2 mL was injected per site on the back of 8 athymic nude mice. Tumor growth was measured weekly using calipers. The tumor area was calculated according to the following formula: tumor area = $\pi \times$ (width/2 × length/2). When the tumors grew to a certain extent (about 6 mm × 4 mm), tissue was removed and fixed in neutral buffered formalin for histological examination. Some cells from the xenografts were re-established *in vitro* to compare their morphology with that of the original cell line.

In vitro invasion assay

The invasion assay was performed by using 24-well BD BioCoat™ Matrigel™ Invasion Chamber with 8-μm polycarbonated filters (Becton Dickinson, Bedford, MA), as described by Albini *et al*[21]. 1×10^4 cells were seeded on Matrigel invasion chamber plates and cultured in routine medium. Cells were incubated for 22 h at 37℃ in a humidified incubator with 5% CO_2. Nonmigratory cells on the upper surface of the filter were removed by wiping with a cotton swab. Invasive cells that penetrated through pores and migrated to the underside of the membrane were stained with Giemsa solution after fixation with 4% formaldehyde in PBS. The cell number was counted under microscopic vision.

Screening of differentially expressed genes

Total RNA was extracted using standard Trizol RNA isolation protocol (Life Technologies, Inc., Grand Island, NY). The poly (A) mRNA was isolated from the total RNA using a poly (dT) resin (Qiagen, Hilden, Germany). Nylon membrane-based cDNA microarrays were prepared by using Spotter (BioRobotics, Cambridge, U.K.) containing clones from the present work and other tissue resources and the internal controls. The procedures for probe preparation, hybridization, washing, scanning, and signal intensity normalization of the spots were performed also using a previously described method[22]. The differential expression was considered as significant between LST and SW480, lovo cell lines when the ratio of signals between the same spots on different membranes was greater than 2.

Western blotting analysis

Western blotting analysis was assessed according to the protocol described before[23]. In brief, cells were washed with ice-cold PBS twice and lysed with ice-cold lysis buffer. Protein samples (15 μg) were separated by SDS/PAGE (12% acrylamide gel) using a Bio-Rad Mini-Protean Ⅲ system (45 mA for about 2 h). Proteins were transferred to PVDF membranes and the membranes were blocked for 1 h at room temperature with TBST. Blots were then incubated at room temperature with primary antibodies (1:500 dilution) and then primary antibodies were removed and the blots were extensively washed with TBS/Tween 20 min for three times. Blots were then incubated for 1 h at room temperature with the secondary antibodies (1:2000 dilution). Then blots were extensively washed as above for 1 h and developed using the Enhanced Chemiluminescence detection system and quantified using the GeneTools systems.

RESULTS

Origin and morphology of LST-R1

When established *in vitro*, LST-R1 cells grew as attached cells with spindle or polygonal morphology. 90% of the individual cells have 1 or 2 heterogenous nuclei with 1 or 2 prominent nucleoli (Figure 2A). To confirm the epithelial origin of this cell line, expression of ESA and CK20 were determined by immunocytochemistry. The positive expression of ESA and CK20 staining revealed that the cells are of epithelial origin (Figure 2 B and C). Under the scanning electron microscope, microvilli and tight junction were observed. There are fewer organelles (such as Golgi apparatus and endoplasmic reticulum) and more secretory granules were observed (Figure 2 D and E).

Cytogenetic analysis

Karyotypic analysis was performed on the LST-R1 cell line at passage 111 by spectral karyotypic technique. The composite karyotypes were summarized in Table 1. Basically, two subclones were identified. Although there were some karyotypic differences between the two subclones, the majority of chromosome aberrations existed in all 30 metaphases analyzed, indicating that the cells were derived from a single progenitor aberrant cell. Chromosome numbers differed in a narrow range (from 64 to 66). A representative karyotype of LST-R1 cells with 66 chromosomes is shown in Figure 2 F.

Origin and morphology of LST-R1

Generally, the cells were triploid with complex structural chromosome aberrations and a number of numerical abnormalities.

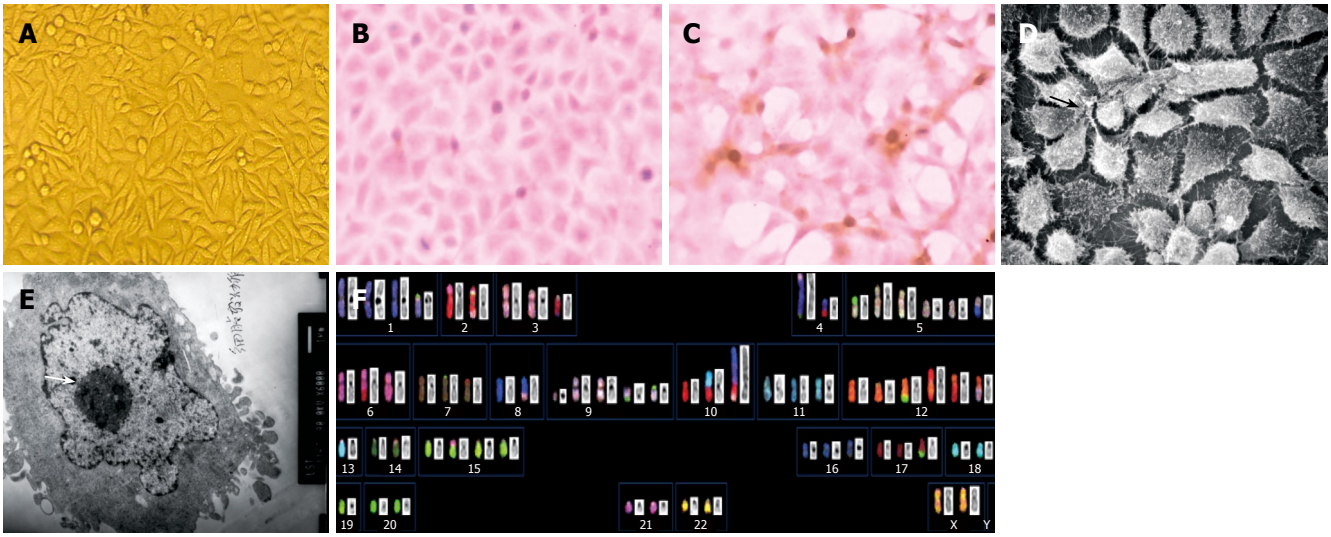


Figure 2 Origin and morphology of LST-R1. **A**: The morphology of cultured cells under microscope at passage 5. The cells are polymorphic and the percentage of epithelial cells is more than 90% (× 10); **B**, **C**: Immunostaining for the epithelial markers ESA and CK20 in LST-R1 cells at passage 5. Cells were grown on chamber slides and stained for ESA (**B**) and CK20 (**C**) expression with a specific antibody; **D**: Under scanning electron microscope (× 1000), dense microvilli and tight junction were observed. Arrow indicates tight junction; **E**: cell observed under transmission electron microscope (× 6000). Arrow indicates nucleus; **F**: Representative karyotype of LST-R1 cell line. Aberrations were observed in the majority of LST-R1 cells. The karyotypic description is listed in Table 1.

Table 1 Chromosome aberrations detected by SKY in LST-R1 cell line

64-66,XX,+der(1)t(1;3)(p11;?)t(3;19)(?;?)[21],der(2)t(2;6)(p11;q23)[30],der(2)t(2;22)(p21;?)t(3;22)(?;?)t(2;3)(?;?)[9],der(2)t(2;22)(p21;?)t(3;22)(?;?)t(2;3)(?;?)t(2;3)(q24;?)[21],-2[30],del(3)(p24)[30],-3[9],der(3)del(p12)t(2;3)(q21;q11)[21],-3[9],der(4)t(4;10)(q11;p11)[30],der(4)dup(14)(q22q31)t(4;14)(q?;q?)[9],der(4)t(3;4)(p21;p16)dup(14)(q22q31)t(4;14)(q?;q?)[21],-4[30],der(5;8)(p10;p10)[30],+i(5)(p10)×2[30],+der(5;20)(p10;q10)del(5)(q31)[30],der(6)t(2:6)(p13;q23)[30],der(7)t(7;15)(p22;q?)[30],der(7)t(7;17)(p13;?)[29],der(8)(3;8)(?;q22)[30],der(9)t(9;19)(p11;?)[30],+der(9)t(9;19)(q11;?)[30],+r(9)(p13q13)[29],der(10;13)(q10;q10)[30],der(10)t(4;10)(p11;11)t(4;8)(q31;q13)t(3;10)(p23;q23)[30],der(12)t(3:12)(q24;q11)[30],der(12)t(5;12)(?;p11)[30],+der(12)t(2;12)(q22;q11)[30],+der(12)t(2;12)(p?;p11)t(2;12)(q24;q?)[30],+der(12)t(12;20)(q24;?)[30],-13[30],-14[30],+der(15)t(6;15)(?;p11)[30],der(17)t(15:17)(q25;q24)[30],-18[30],-19[30],-19[30],-21[30],der(22)t(3;22)(?;p13)[9],-22[30][cp30]

The growth properties of LST-R1 in vitro and in vivo

LST-R1 cells were passaged for more than 110 times. In medium containing 10% FBS, the cells grew as monolayers with varying degrees of attachment. They grew rapidly with large nuclei. The population doubling time was 36 h, and the mitotic index arrived at the peak value at the fourth day in a 25-cm^2 flask (Figure 3A).

The *in vivo* tumorigenicity of LST-R1 cells was studied using athymic nude mice xenograft. Tumor formation was observed 15 s after inoculation, and tumors grew in all of the 8 mice. Tumor area progressively increased and reached an average of 3 cm^2 in size 28 s after inoculation. The histopathology of the xenograft tumor showed that it was a poorly differentiated adenocarcinoma (Figure 3B). Cells cultured from the xenograft tumor (using the same protocol as previously described) had the same properties as the original cell line.

The invasion ability of LST-R1 in vitro

In vitro invasion assay revealed that invasive activity increased significantly in LST-R1 comparing with the other two cell lines. Twenty-four hours later, the numbers of LST-R1 cells invading the membrane were significantly higher (179.3 ± 40 cells/well) than those of lovo (139.3 ±

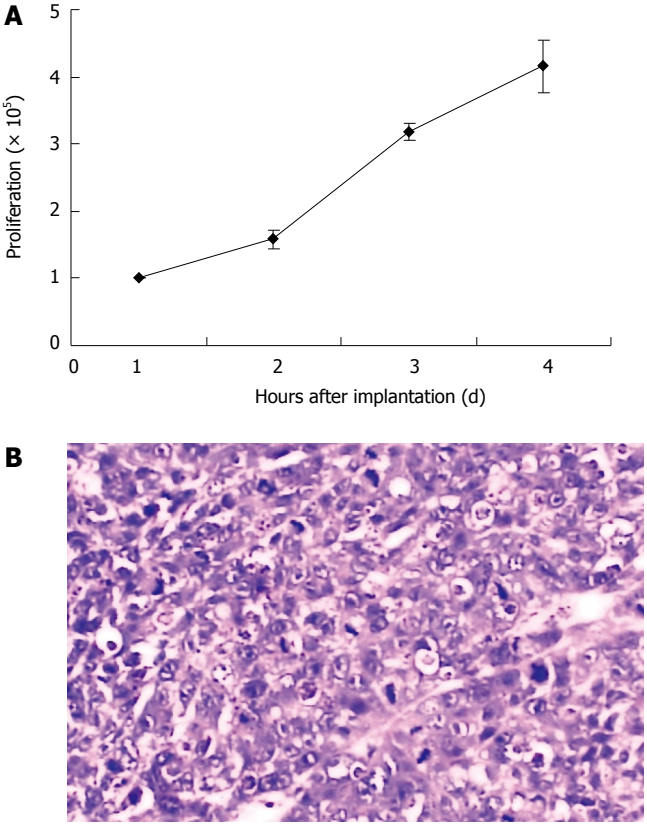


Figure 3 The growth properties of LST-R1 *in vitro* and *in vivo*. **A**: The growth curve of LSR-R1 cell line. Data points are the means of three independent experiments; **B**: Tumorigenicity *in vivo*. The nude mice were sacrificed when the tumor reached 60 mm × 40 mm in size. The histopathology shows a poor differentiated adenocarcinoma.

Table 2 Genes differentially expressed in LST cells when compared with SW480 cells are functionally categorized based on the Gene Ontology terms available from the online batch extract interface for SOURCE. (http://smd.stanford.edu/cgi-bin/source/sourceBatchSearch). This table shows the genes categorized in cell adhesion

Accession	Gene name	Gene symbol	Fold change
Z24725	Mitogen inducible 2	MIG2	3.21
Z22555	CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 1	CD36L1	3.10
S80990	Ficolin (collagen/fibrinogen domain-containing) 1	FCN1	2.91
AL137480	Formin binding protein 4	FNBP4	2.73
AL117505	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	ATP2A2	2.63
M26683	Small inducible cytokine A2 (monocyte chemotactic protein 1, homologous to mouse Sig-je)	SCYA2	2.45
AF055084	Monogenic, audiogenic seizure susceptibility 1 homolog (mouse)	MASS1	2.39
X68264	Melanoma cell adhesion molecule	MCAM	2.38
AF052124	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I , early T-lymphocyte activation 1)	SPP1	2.31
M25280	Selectin L (lymphocyte adhesion molecule 1)	SELL	2.23
M16336	CD2 antigen (p50), sheep red blood cell receptor	CD2	2.19
AL161973	Myeloid/lymphoid or mixed-lineage leukemia [trithorax (Drosophila) homolog]; translocated to, 4	MLLT4	2.14
L25286	Collagen, type XV, alpha 1	COL15A	2.10
D83018	Nel (chicken)-like 2	NELL2	-48.1
AB040902	Fibronectin leucine rich transmembrane protein 3	FLRT3	-3.51
AA460440	CD164 antigen, sialomucin	CD164	-2.23
U85611	Calcium and integrin binding protein (DNA-dependent protein kinase interacting protein)	CIB1	-2.03

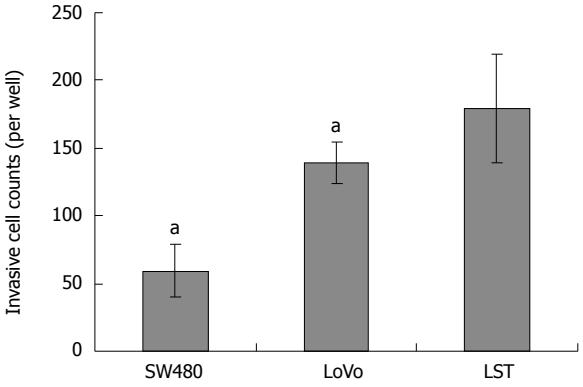


Figure 4 The invasion abilities of various cancer cell lines. The Matrigel invasion chamber was used to measure the LST cells invading through a matrix of reconstituted basement membrane compared to the other two cell lines SW480 and lovo. $^{a}P < 0.05$, significant difference between LST and other two cell lines, paired *t*-test.

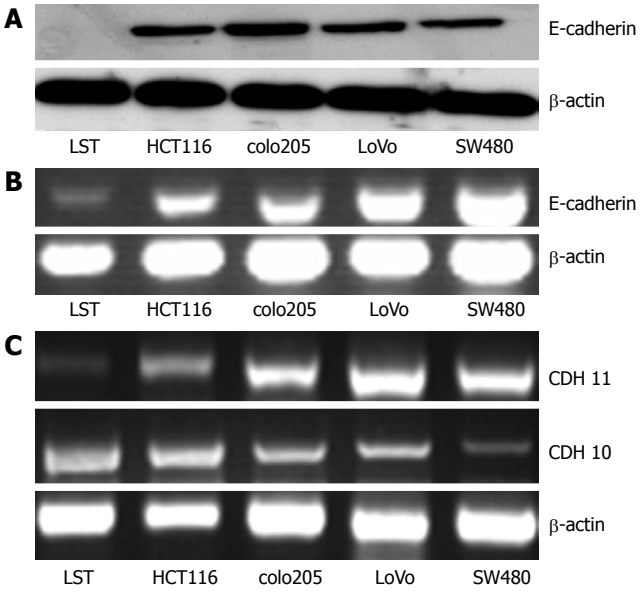


Figure 5 Detection of E-cadherin, CDH10 and CDH11 expression in various colorectal cancer cell lines by Western blotting analysis (**A**) and RT-PCR (**B** and **C**). The experiment was repeated for three times with similar result as shown here.

15.1 cells/well) and SW480 (59.3 ± 19.1 cells/well) cells (Figure 4).

cDNA microarray analysis

As shown in Tables 2 and 3, the cDNA microarray was applied to analyse the expression patterns of the three cell lines: SW480, lovo, LST. Expression of the cell adhesion molecule E-cadherin in different colon cancer cell lines was confirmed by Western blotting. As shown in Figure 5, the expression of E-cadherin in LST cell line was at a very low level comparing with the other four colon cancer cell lines.

DISCUSSION

Recent molecular studies have demonstrated a different histogenesis of flat and depressed tumor in comparison with polypoid adenomas. Flat and depressed tumor has a more malignant tendency and LST is an important subtype of it[24-27]. In the study of disease genesis, cell line and animal model are useful tools to understanding the biological characteristics. However, until now, there is no report on LST cell line and/or animal model. So there is an absolute need to establish *in vitro* model systems to study the distinctive behavior of LST and the potential mechanism of colon carcinogenesis.

In the present study, we report the characterization of LST-R1 from a fresh laterally spreading tumor specimen. Although the tissue, which the cell line derived from, is villous adenoma accompanied by severe atypical hyperplasia, our data showed it has an obvious malignant tendency. First, LST-R1 cells were passaged more than 110 times without the use of any immortalizing agent;

Table 3 Genes differentially expressed in LST cells when compared with LoVo cells are functionally categorized based on the Gene Ontology terms available from the online batch extract interface for SOURCE. (http://smd.stanford.edu/cgi-bin/source/sourceBatchSearch). This table shows the genes categorized in cell adhesion

Accession	Gene name	Gene symbol	Fold change
S80990	Ficolin (collagen/fibrinogen domain-containing) 1	FCN1	42.87
AL161973	Myeloid/lymphoid or mixed-lineage leukemia (trithorax (Drosophila) homolog); translocated to, 4	MLLT4	26.47
M26683	Small inducible cytokine A2 (monocyte chemotactic protein 1, homologous to mouse Sig-je)	SCYA2	24.92
M31210	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 1	EDG1	11.07
Z22555	CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 1	CD36L1	4.33
M16336	CD2 antigen (p50), sheep red blood cell receptor	CD2	3.07
U43901	Laminin receptor 1 (67 kD, ribosomal protein SA)	LAMR1	2.55
M85289	Heparan sulfate proteoglycan 2 (perlecan)	HSPG2	2.11
Y13323	ADAM-like, decysin 1	ADAMDEC1	-2.03
AL133035	Filamin-binding LIM protein-1	FBLP1	-2.10
NM_002292	Laminin, beta 2 (laminin S)	LAMB2	-2.10
AL161972	Intercellular adhesion molecule 2	ICAM2	-2.12
BE301622	Pinin, desmosome associated protein	PNN	-2.17
AF053944	AE-binding protein 1	AEBP1	-2.21
Y10931	Symplekin	SYMPK	-2.24
M98457	CD209 antigen	CD209	-2.25
D87469	Cadherin, EGF LAG seven-pass G-type receptor 2, flamingo (Drosophila) homolog	CELSR2	-2.26
AW843882	Leucine-rich repeats and immunoglobulin-like domains 3	LRIG3	-2.26
X78947	Connective tissue growth factor	CTGF	-2.33
AF135021	Catenin (cadherin-associated protein), alpha-like 1	CTNNAL1	-2.36
AL136139	Enhancer of filamentation 1 (cas-like docking; Crk-associated substrate related)	HEF1	-2.36
AF055084	Monogenic, audiogenic seizure susceptibility 1 homolog (mouse)	MASS1	-2.37
AF039747	Cadherin 10, type 2 (T2-cadherin)	CDH10	-2.39
U58516	Milk fat globule-EGF factor 8 protein	MFGE8	-2.41
D49742	Hyaluronan-binding protein 2	HABP2	-2.42
U79716	Reelin	RELN	-2.42
Y11307	Cysteine-rich, angiogenic inducer, 61	CYR61	-2.45
AB007865	Fibronectin leucine rich transmembrane protein 2	FLRT2	-2.48
NM_006149	Lectin, galactoside-binding, soluble, 4 (galectin 4)	LGALS4	-2.52
M59807	Natural killer cell transcript 4	NK4	-2.59
AJ009985	Annexin A9	ANXA9	-2.61
NM_014000	Vinculin	VCL	-2.63
AL117505	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	ATP2A2	-2.64
AF062075	Leupaxin	LPXN	-2.67
X05610	Collagen, type Ⅳ, alpha 2	COL4A2	-2.70
D12676	CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 2 (lysosomal integral membrane protein Ⅱ)	CD36L2	-2.72
D21255	Cadherin 11, type 2, OB-cadherin (osteoblast)	CDH11	-2.77
U70136	Proteoglycan 4, (megakaryocyte stimulating factor, articular superficial zone protein)	PRG4	-2.77
S53911	CD34 antigen	CD34	-2.82
AW025274	Kin of IRRE like 2 (Drosophila)	KIRREL2	-2.85
M73255	Vascular cell adhesion molecule 1	VCAM1	-2.86
L25286	Collagen, type XV, alpha 1	COL15A1	-2.87
AL117604	Deleted in liver cancer 1	DLC1	-2.91
AJ132445	Claudin 14	CLDN14	-2.95
AL133035	Filamin binding LIM protein 1	FBLP1	-3.14
Z24725	Mitogen inducible 2	MIG2	-3.15
X15880	Collagen, type Ⅵ, alpha 1	COL6A1	-3.53
AL137480	Formin binding protein 4	FNBP4	-3.58
AF052124	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein Ⅰ, early T-lymphocyte activation 1)	SPP1	-3.60
AB018269	Calsyntenin 3	CLSTN3	-3.70
AB002298	PDZ domain containing 3	PDZK3	-3.97
X87838	Catenin (cadherin-associated protein), beta 1 (88 kDa)	CTNNB1	-4.64

second, the karyotypic analysis showed the cells were triploid with structural chromosome aberrations and a number of numerical abnormalities; third, the tissue from the inoculated nude mice are poorly differentiated adenocarcinoma, which suggests the cell line is originated from malignant clones. The most possible reason for this difference could be that the pathologic type of the tissue (60 mm × 70 mm) was large and uneven, and only a little part of it has been taken for histopathological study while most of the tissue was taken for cell culture. Analysis from only a small number of serial sections might lead to the missed diagnosis of the cancer. Normal cells proliferate at a slower rate than the malignant cells, therefore only the malignant cells left after several passages.

The epithelial and colorectal origin of these cells were confirmed by positive ESA and CK20 staining, miniville and tight junction observed under the electron microscope, as well as morphological observation. After 15 d of inoculation in nude mice, the tumors occurred and grew vertically rather than laterally spreading. The reason

we proposed is associated with the different circumstances between hypodermic and colon lumen mucosa. Although the lack of lateral growth in nude mice limits its utility as an *in vivo* model, it may prove otherwise as an *in vitro* model system to investigate biological behaviors and malignancy-associated phenotypes of the disease at its onset.

The superficial colon tumor is more likely to invade the mucous membranes and developed cancer[2]. So we compared invasion ability between LST-R1 and other two colorectal cancer cell lines derived from prudent colon cancer with the Matrigel invasion chamber. SW480 is human colorectal adenocarcinoma (Duke' type B). Lovo is human colorectal adenocarcinoma derived from a fragment of a metastatic tumor nodule in the left supraclavicular region (Duke' type C, grade Ⅳ). Our results demonstrated that the number of LST cells invading through the membrane was more than the other two cell lines and showed that the LST-R1 cells had a high ability of invasion, which was in consistent with the high malignancy of LST.

It is well known that the low expression of the adhesion function contributes to the increasing invasive ability of colon cancer. To explore the reason of higher invasion of LST-R1 cell line, we compared the gene expression profile of LST-R1 cells with SW480 and lovo cells by cDNA microarray. According to cDNA microarray data, a number of genes associated with cell adhesion changed, such as E-cadherin. E-cadherin is a 120-kDa transmembrane glycoprotein that belongs to a large superfamily of calcium-dependent cadherin adhesion molecules[27] and plays an important role in embryogenesis and in mature epithelia. Abnormalities of E-cadherin expression have been described in colorectal carcinoma[28] and have been correlated with increased invasiveness, metastatic potential and poor patient prognosis in this tumor type[29]. Recent research showed that E-cadherin expression was significantly lower in the de novo group tissue than that in the prudent group[30,31]. In the present study, by Western blotting analysis, we also confirmed that LST-R1 cells have a prominent lower expression of E-cadherin than that in several other cell lines. Our data therefore suggest that the high invasive ability of LST-R1 cell line has a close relationship with the low expression of adhesion molecule. Further investigations should be taken to study the role of the adhesion molecules (such as E-cadherin) in determining the malignancy of LST.

In conclusion, we established a stable cell line (LST-R1) derived from an early colon cancer-LST and this cell line has an obvious malignant tendency. LST-R1 is also characterized with high invasive ability, and this property may result from the loss of adhesion molecules such as E-cadherin.

COMMENTS

Background

Invasion is an important characteristic of malignant tumor and the mechanism of invasion is not very clear. Many genes had been verified to be involved in invasion of cancer including E-cadherin. Cell lines are useful tools for molecular level study and many cell lines have been established for various studies.

Research frontiers

Laterally spreading tumor (LST) is a unique subtype of superficial colorectal tumor and in some studies, it has been reported that it had a higher carcinogenesis rate. Invasion is a pivotal step of colorectal cancer carcinogenesis progression and E-cadherin is an important invasion-associated gene. We established a colorectal cell line with high invasion ability and a low expression of E-cadherin.

Innovations and breakthroughs

In this study, we establish a cell line derived from a laterally spreading tumor tissue. Our data suggested that this cell line is a colorectal cell line and has high invasion ability compared with SW480 and Lovo cell lines. There is a loss of E-cadherin expression in Laterally Spreading Tumor-Rectum 1 (LST-R1) cells. This cell line will be a useful tool for the study of colorectal cancer invasion mechanism.

Applications

This cell line is a useful tool for colorectal cancer invasion mechanism study. We will investigate the role of E-cadherin in LST-R1 cells and the relationship between low expression of E-cadherin and high invasion ability.

Terminology

SKY in this study means metaphase chromosome preparation and spectral karyotyping.

Peer review

This article is interesting. In this article, the authors characterized a laterally spreading tumor cell line and compare it to several typical colorectal cancer cell lines. Since these tumors progress in a different fashion than typical adenoma to carcinoma colorectal tumors that protrude into the gut lumen, the isolation and comparison of expression of growth patterns of these cells is important.

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CLINICAL RESEARCH

Highly metabolic thrombus of the portal vein: ^{18}F fluorodeoxyglucose positron emission tomography/computer tomography demonstration and clinical significance in hepatocellular carcinoma

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Abstract

AIM: To assess the ability of ^{18}F-fluorodeoxyglucose positron emission tomography/computer tomography (^{18}F-FDG PET/CT) to differentiate between benign and malignant portal vein thrombosis in hepatocellular carcinoma (HCC) patients.

METHODS: Five consecutive patients who had HBV cirrhosis, biopsy-proven HCC, and thrombosis of the main portal vein and/or left/right portal vein on ultrasound (US), computer tomography (CT) or magnetic resonance imaging (MRI) were studied with ^{18}F-FDG PET/CT. The presence or absence of a highly metabolic thrombus on ^{18}F-FDG PET/CT was considered diagnostic for malignant or benign portal vein thrombosis, respectively. All patients were followed-up monthly with US, CT or MRI. Shrinkage of the thrombus or recanalization of the vessels on US, CT or MRI during follow-up was considered to be definitive evidence of the benign nature of the thrombosis, whereas enlargement of the thrombus, disruption of the vessel wall, and parenchymal infiltration over follow-up were considered to be consistent with malignancy. ^{18}F-FDG PET/CT, and US, CT or MRI results were compared.

RESULTS: Follow-up (1 to 10 mo) showed signs of malignant thrombosis in 4 of the 5 patients. US, CT or MRI produced a true-positive result for malignancy in 4 of the patients, and a false-positive result in 1. ^{18}F-FDG PET/CT showed a highly metabolic thrombus in 4 of the 5 patients. ^{18}F-FDG PET/CT achieved a true-positive result in all 4 of these patients, and a true-negative result in the other patient. No false-positive result was observed using ^{18}F-FDG PET/CT.

CONCLUSION: ^{18}F-FDG PET/CT may be helpful in discriminating between benign and malignant portal vein thrombi. Patients may benefit from ^{18}F-FDG PET/CT when portal vein thrombi can not be diagnosed exactly by US, CT or MRI.

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Key words: ^{18}F-fluorodeoxyglucose; Positron emission tomography/computer tomography; Hepatocellular carcinoma; Portal vein tumor thrombus; Portal vein blood thrombus

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INTRODUCTION

Hepatocellular carcinoma (HCC) carries a high risk of invasion of the portal vein. The detection and etiologic characterization of these thrombi are essential for treatment planning, particularly in patients with hepatic tumors, because malignant thrombosis is a negative factor in terms of prognosis[1,2]. Macroscopic tumor thrombi in the portal vein appear to occur during the terminal stage of HCC. The management of HCC with portal vein tumor thrombosis is complicated and controversial[3,4]. However, benign portal vein thrombi can usually be successfully

managed with early identification[5]. Portal vein thrombosis (PVT) can be diagnosed noninvasively by using either real-time or duplex Doppler sonography, contrast-enhanced sonography, computer tomography (CT) or magnetic resonance imaging (MRI). Typical appearances of venous thrombosis include an intraluminal filling defect, peripheral ring-like enhancement and collateral venous channels[6,7]. Micro-invasive fine-needle aspiration (FNA) of a portal vein thrombus under ultrasound guidance also is an effective, well-tolerated method for the diagnosis of HCC patients with PVT[8,9].

This pattern of ^{18}F-FDG PET/CT has been reported in cases with different kinds of malignant tumor[10], while portal tumor growth and portal vein thrombosis identified by PET/CT in HCC patients have rarely been reported to date. We found highly metabolic thrombi of the portal vein on ^{18}F-FDG PET/CT scans in four patients with HCC. ^{18}F-FDG -avid lesions may represent malignant portal vein thrombosis. The importance of this finding for the diagnosis of and prognosis in HCC and the role of ^{18}F-FDG PET/CT in the diagnosis of PVT are discussed.

MATERIALS AND METHODS

Patients

Between January 2007 and October 2007, five HCC patients with suspected portal vein tumor thrombus (PVTT) were assigned to PET/CT evaluation. Five consecutive patients who had HBV cirrhosis, biopsy-proven HCC, and thrombosis of the main portal vein and/or left/right portal vein on US, CT or MRI, were also studied by ^{18}F-FDG PET/CT. PET/CT. US, CT or MRI results were compared with those at follow-up.

FDG PET/CT technique

The patients were asked to fast for at least 4 h before undergoing PET/CT. The blood glucose levels of these patients should have been within the normal range (0.7-1.2 g/L) prior to intravenous injection of ^{18}F-FDG. The patients received an intravenous injection of 370-666 MBq (10-18 mCi) of ^{18}F-FDG. Data acquisitions by an integrated PET/CT system (Discovery STE; GE Medical Systems, Milwaukee, WI, USA) were performed within 60 min after the injection. Data acquisition was performed as follows: CT scanning was performed first, from the head to the pelvic floor, with 110 kV, 110 mA, a tube rotation time of 0.5 s and a 3.3 mm section thickness, which was matched to the PET section thickness. Immediately after CT scanning, a PET emission scan that covered an identical transverse field of view was obtained. Acquisition time was 3 min per table position. PET image data sets were reconstructed iteratively by applying the CT data for attenuation correction, and co-registered images were displayed on a workstation.

Definitive diagnoses of thrombosis and thrombus malignancy

On integrated PET/CT images, thrombi were considered malignant if the maximum standardized uptake value (SUV) was greater than those of normal liver structures with a discrete margin and/or the ^{18}F-FDG uptake was greater than that of the lumen of the descending aorta in the same axial slice. Thrombi were considered benign if the maximum SUV was lower than those of normal liver structures and/or the ^{18}F-FDG uptake was lower than that of the lumen of the descending aorta in the same slice.

All patients were followed-up monthly by US, CT or MRI. Shrinkage of the thrombus and/or recanalization of the vessels on US, CT or MRI during follow-up was considered to be definitive evidence of the benign nature of the thrombosis, whereas enlargement of the thrombus, disruption of the vessel wall, and parenchymal infiltration over follow-up were considered to be consistent with malignancy.

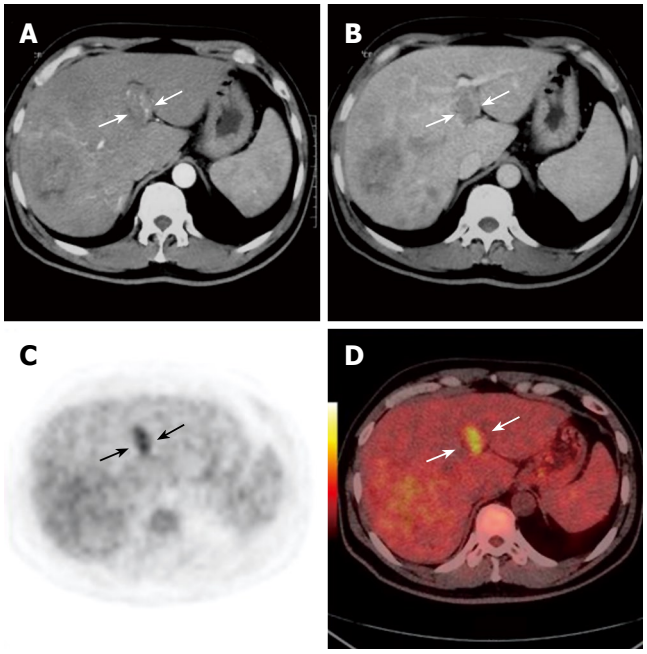


Figure 1 Contrast CT scan showing right lobe and caudate lobe mass and portal vein thrombus in a 55-year-old man. During the arterial phase, the thrombus appears as an iso- to hypodense intraluminal area with dense linear enhancement (white arrows, **A**). A filling defect was detected in the left branch during the portal phases (white arrows, **B**). PET (black arrows, **C**) and PET/CT fused images (white arrows, **D**) reveal a highly metabolic thrombus in the left branch.

RESULTS

Follow-up (1 to 10 mo) showed signs of malignant thrombosis in 4 out of 5 patients. US, CT or MRI produced a true-positive result for malignancy in 4 out of 5 patients, and a false-positive result in 1. ^{18}F-FDG PET/CT was positive in four patients with malignant thrombi, and revealed a true-negative result in the remaining patient. ^{18}F-FDG PET/CT showed highly metabolic thrombi in 4 of the 5 patients. No false-positive result was obtained using ^{18}F-FDG PET/CT.

As shown in Figures 1-5, the four malignant thrombi had SUVs of 3.0-11.5, which were higher than those of the normal liver parenchyma and the lumen of the descending aorta in the same axial slice. The SUVs of these malignant thrombi were lower than those of the HCC mass in the same axial slice. Thrombosis was significantly more common in patients with highly metabolic tumor lesions

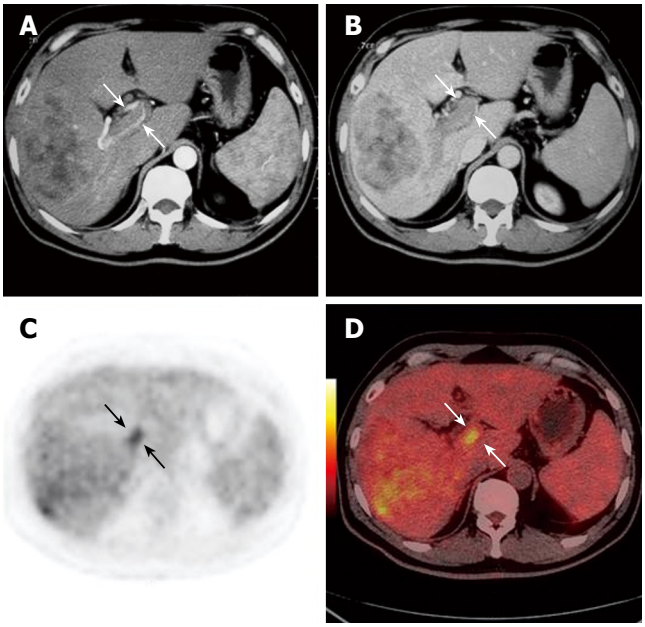


Figure 2 Contrast CT scan showing right lobe and caudate lobe mass and portal vein thrombus of the right branch in the same patient. During the arterial phase, the thrombus appears as an iso- to hypodense intraluminal area with dense linear enhancement (white arrows, **A**). A filling defect was detected in the right branch during the portal phases (white arrows, **B**). PET (black arrows, **C**) and PET/CT fused images (white arrows, **D**) reveal a highly metabolic thrombus in the left branch.

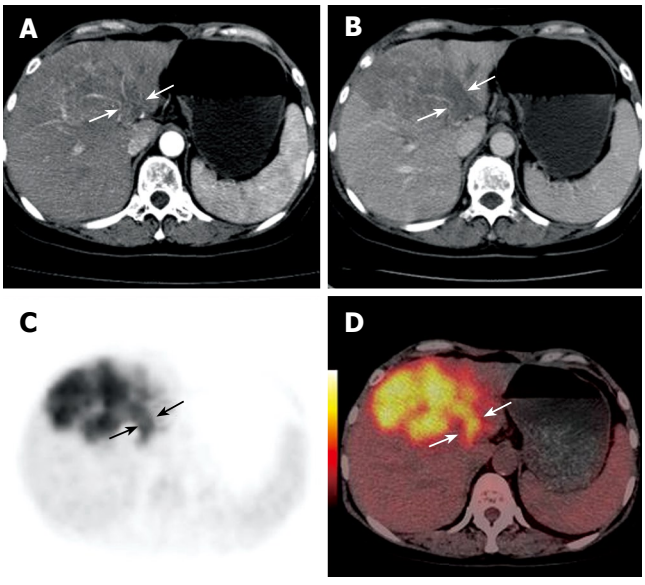


Figure 3 Contrast CT scan demonstrating a left lobe mass and portal vein thrombus in a 60-year-old woman. During the arterial phase, the thrombus appears as a hypodense intraluminal area with dense linear enhancement (white arrows, **A**). A filling defect was detected in the left branch during the portal phases (white arrows, **B**). PET (black arrows, **C**) and PET/CT fused images (white arrows, **D**) reveal a highly metabolic thrombus in the left branch.

than in those with weakly metabolic tumor lesions. All four patients with malignant thrombi had advanced HCC. All of the malignant high metabolism thrombi had discrete margins in PET and PET/CT images.

The remaining patient was shown by ultrasound to have a portal thrombus in the left portal vein 6 mo after HCC

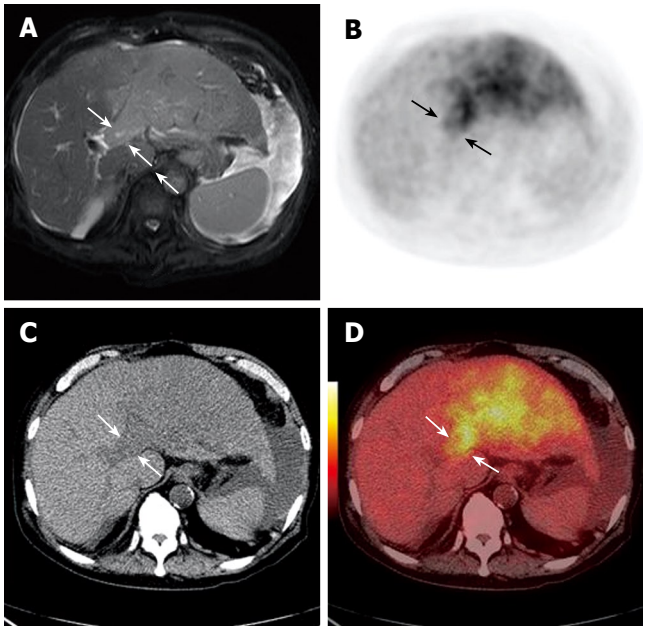


Figure 4 MRI scan showing a left lobe mass and portal vein thrombus in an 80-year-old man (white arrows, **A**). CT detected a low-density lesion in the left branch (white arrows, **C**). PET (black arrows, **B**) and PET/CT fused images (white arrows, **D**) reveal a highly metabolic thrombus in the left branch.

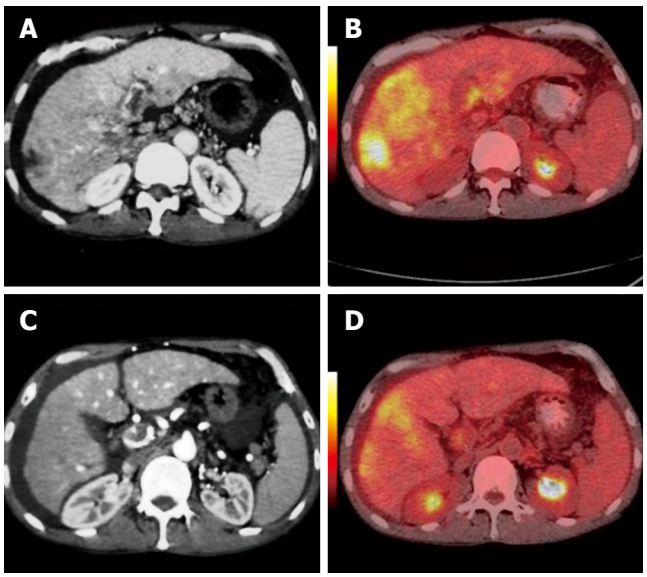


Figure 5 Contrast CT scan showing a diffuse tumor and portal vein thrombus in a 44-year-old man. During the arterial phase, the thrombus appears as a hypodense intraluminal area with mild enhancement at the LV (white arrows, **A**) and PV (white arrows, **C**). PET/CT fused images reveal a highly metabolic mass and a highly metabolic thrombus at the LV and PV, respectively (white arrows, **B**, **D**).

resection. Malignant thrombus was suspected on contrast CT. Contrast-enhanced MRI demonstrated a thrombus in the left portal vein. In the PET/CT examination, the thrombus of the left portal vein had an SUV of 3.0, which was lower than those of the normal liver parenchyma and lumen of descending aorta. ^{18}F-FDG PET/CT found no high ^{18}F-FDG uptake in the left portal vein. The left portal vein was recanalized in the contrast MRI 10-mo follow-up (Figure 6). Finally, diagnosis of the left portal vein blood

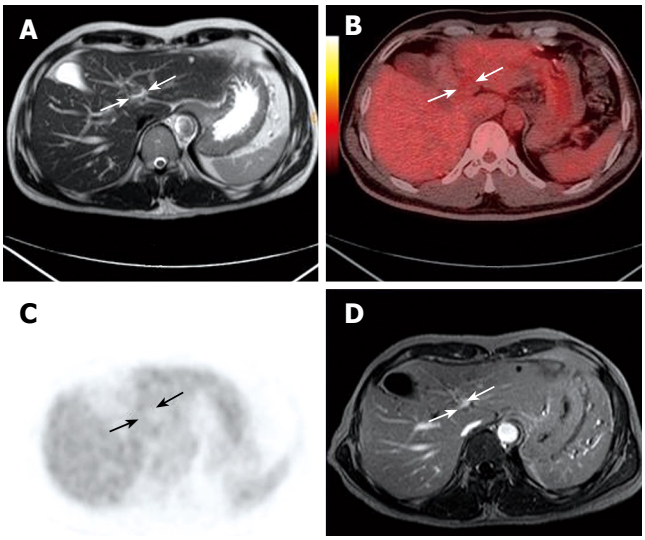


Figure 6 MRI showing a portal vein thrombus in a 44-year-old man six months after HCC resection (white arrows, **A**). PET/CT fused images (white arrows, **B**) and PET (black arrows, **C**) reveal no highly metabolic thrombus in the left branch. LP was recanalized in the contrast MRI at 10 mo follow-up (white arrows, **D**).

thrombus was established. Tables 1 and 2 summarize the clinical and image characteristics of the five patients.

DISCUSSION

HCC tends to invade the intrahepatic vasculature, especially the portal vein. In clinically treated series, the rate of portal invasion is 34%-40%[11-13]. The natural history of untreated HCC is still poor, especially in patients with PVTT; the median survival of such patients was reported to be 2.7 mo, whereas survival in those without PVTT was 24.4 mo. PVTT causes acute portal hypertension, acute upper gastrointestinal hemorrhage, refractory ascites, and finally, acute liver function failure[14-16]. Our findings in the present study confirm portal vein thrombosis is a frequent complication of primary liver tumors, especially those with diameters larger than 3.0 cm, and diffuse tumors.

The reported increased sensitivity of PET/CT over CT has been attributed to the ability of FDG-PET to detect metabolic abnormalities that precede the morphologic changes seen by CT. The global (skull base to proximal thighs) nature of PET/CT study also contributes to increased sensitivity through the detection of distant metastases[17]. ^{18}F-FDG PET/CT has become one of the standard imaging modalities in diagnosing and staging of tumors, and monitoring therapeutic efficacy in hepatic malignancies[18-19]. In this initial clinical experience, ^{18}F-FDG PET/CT appears to be a reliable technique for evaluating the veins of the portal system thrombus. The main advantage of ^{18}F-FDG PET/CT over conventional sonography techniques is its ability to assess the ^{18}F-FDG metabolism activity of portal vein thrombi. Compared with US, the main limitations of no contrast ^{18}F-FDG PET/CT is the absence of ability to observe the portal vein blood stream clearly along the main portal vein and its branch[20,21]. In the fourth patient, arterioportal shunt was confirmed by contrast CT. Thus, when ^{18}F-FDG PET/CT-suspected PVTT is considered, a whole body ^{18}F-FDG PET/CT examination combined with a contrast three-phase liver CT scan in the same PET/CT system is recommended.

Table 1 Clinical data on patients with high and low-metabolism thrombi of the portal vein

Patient	Age (yr)/ sex	Symptoms and signs	Laboratory data	Outcome
1	55/M	No	AFP 5 ng/mL; abnormal HFT	Died of liver failure 2 mo after EPI
2	60/F	Pain in right hypochondriac region	AFP > 1000 ng/mL; abnormal HFT	Died of hepatic failure 3 mo after TACE
3	80/M	Cough	AFP 30 ng/mL; abnormal HFT	Died of renal failure 1 mo after admission
4	65/M	Ascites	AFP > 1000 ng/mL; abnormal HFT	Died of liver failure 1 mo after admission
5	44/M	Six mo after HCC resection	AFP normal; normal HFT	Recanalization

AFP: Alpha-fetoprotein level; HFT: Hepatic function test.

Table 2 Imaging data on patients with high and low-metabolism thrombi of the portal vein

Patient	US, CT, MRI findings		FDG PET/CT SUV ratio		
	Liver tumor location and size	Location of PV tumor thrombus	T/NT	Th/NT	Th/DAo
1	Right and caudate lobe 11 cm × 13 cm	RPV LPV	4.8/3.0 4.8/5.9	5.2/3.0 5.9/3.0	5.2/2.1 5.9/2.1
2	Right lobe 8.4 cm × 9.6 cm	LPV	15.5/2.8	11.5/2.8	11.5/2.7
3	Left lobe diffuse tumor	LPV	9.0/3.6	7.9/3.6	7.9/2.4
4	Both lobes diffuse tumor	LPV PV	5.3/2.7 5.3/2.7	4.9/2.7 3.4/2.7	4.9/2.2 3.4/2.2
5	Six mo after right lobe lesion resection	LPV	-	3. 0/3.0	3.0/2.0

SUV: Standardized uptake value; T: Tumor target; NT: Non tumor target; Th: Thrombi; Dao: Descending aorta; RPV: Right portal vein; LPV: Left portal vein; PV: Portal vein.

Preliminary studies have already highlighted the potential value of ^{18}F-FDG PET/CT in HCC staging and restaging for the TNM stage. It is helpful in early diagnosis and proper treatment, which are very important for patients with PVTT to prolong their lives[22-24]. The reported increased sensitivity of PET/CT over CT has been attributed to the ability of ^{18}F-FDG PET to detect metabolic abnormalities that precede the morphologic changes seen by CT. The global (skull base to proximal thighs) nature of PET/CT study also contributes to increased sensitivity through the detection of distant metastases in HCC patients[25,26].

Dodd *et al*[27] reported that emboli might develop in the portal veins of hepatocirrhosis patients, and that these are mostly portal vein blood thrombi (PVBT), benign hepatic cells, or even bile duct epithelial cells. In recent years, some scholars have found that, during percutaneous ethanol injection, the alcohol that leaks out of the tumor and

flows into the portal vein may cause a benign thrombus. ^{18}F-FDG is not only uptaken by tumor cells, but also by normal cells[28-31] because benign hepatic cells and even bile duct epithelial cells in PVBT can uptake ^{18}F-FDG, which may also have active metabolism in PET/CT images. Thus, further studies are needed to confirm the ability of ^{18}F-FDG PET to discriminate PVTT and PVBT. The potential of this novel approach to characterize the veins of a portal system thrombus should be elucidated in large, ongoing clinical trials.

In conclusion, ^{18}F-FDG PET/CT can be helpful in discriminating between benign and malignant portal vein thrombi. Patients may benefit from ^{18}F-FDG PET/CT when portal vein thrombi can not be diagnosed exactly by US, CT or MRI.

COMMENTS

Background

To select an appropriate treatment regimen, it is essential to accurately characterize the nature of a thrombus in hepatocellular carcinoma (HCC) patients. This study investigated the ability of ^{18}F-fluorodeoxyglucose positron emission tomography/computer tomography (^{18}F-FDG PET/CT) to differentiate between benign and malignant portal vein thrombosis in HCC patients.

Research frontiers

PET and CT are becoming more and more widely available, and their application with ^{18}F-FDG in oncology is beginning to be realized. However, the potential for ^{18}F-FDG PET/CT in the diagnosis of benign and malignant portal vein thrombosis in HCC patients is undefined.

Innovations and breakthroughs

Thrombi with maximum standardized uptake values (SUVs) greater than those of normal liver structures with a discrete margin and/or an ^{18}F-FDG uptake greater than that of the lumen of the descending aorta in the same axial slice were considered malignant. Thrombi with maximum SUVs lower than those of normal liver structures and/or an ^{18}F-FDG uptake lower than that of the lumen of the descending aorta in the same slice were considered benign.

Applications

Our findings can be helpful in discriminating between benign and malignant portal vein thrombi, and can be used to select an appropriate treatment for HCC patients, especially when portal vein thrombi can not be diagnosed exactly by US, CT or MRI.

Peer review

In this study, the authors reported the clinical significance of ^{18}F-FDG PET/CT for the discrimination between benign and malignant thrombi in advanced HCC. This manuscript arouses interest for readers and provides an important clue to diagnose or differentiate portal thrombus characteristics.

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RAPID COMMUNICATION

Diagnosis of mild chronic pancreatitis (Cambridge classification): Comparative study using secretin injection-magnetic resonance cholangiopancreatography and endoscopic retrograde pancreatography

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Abstract

AIM: To investigate the usefulness of secretin injection-MRCP for the diagnosis of mild chronic pancreatitis.

METHODS: Sixteen patients having mild chronic pancreatitis according to the Cambridge classification and 12 control subjects with no abnormal findings on the pancreatogram were examined for the diagnostic accuracy of secretin injection-MRCP regarding abnormal branch pancreatic ducts associated with mild chronic pancreatitis (Cambridge Classification), using endoscopic retrograde cholangiopancreatography (ERCP) for comparison.

RESULTS: The sensitivity and specificity for abnormal branch pancreatic ducts determined by two reviewers were respectively 55%-63% and 75%-83% in the head, 57%-64% and 82%-83% in the body, and 44%-44% and 72%-76% in the tail of the pancreas. The sensitivity and specificity for mild chronic pancreatitis were 56%-63% and 92%-92%, respectively. Interobserver agreement (κ statistics) concerning the diagnosis of an abnormal branch pancreatic duct and of mild chronic pancreatitis was good to excellent.

CONCLUSION: Secretin injection-MRCP might be useful for the diagnosis of mild chronic pancreatitis.

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Key words: Magnetic resonance cholangiopancreatography; Endoscopic retrograde cholangiopancreatography; Mild; Chronic pancreatitis; Diagnosis

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INTRODUCTION

Chronic pancreatitis is morphologically characterized by irregular sclerosis associated with destruction and permanent loss of the exocrine parenchyma which may be either, focal, segmental or diffuse. These changes may present together with varying degrees of dilatation of the duct system at different segments[1]. Chronic pancreatitis is classified according to morphologic changes of the pancreatic ducts, as defined in the 1983 Cambridge Symposium[2].

MR cholangiopancreatography (MRCP) is a useful non-invasive technique to depict the projection images of the pancreatico-biliary tree, and is expected to become a valuable alternative to endoscopic retrograde cholangiopancreatography (ERCP)[3-8]. However, visualization of the branch pancreatic ducts by MRCP is not good[9-11], and there are few reports about the diagnostic value of MRCP in mild chronic pancreatitis (Cambridge classification). On the other hand, secretin injection-MRCP might allow better visualization of the pancreatic ductal system[12-16].

The purpose of this study was to evaluate the usefulness of secretin injection-MRCP for the diagnosis of abnormal branch pancreatic duct in patients with mild chronic pancreatitis.

MATERIALS AND METHODS

Patients

The MRCP image obtained after secretin injection was examined in 16 patients (32-39 y.o.; mean 36 y.o.) having

mild chronic pancreatitis according to the Cambridge classification, and in 12 control subjects (31-37 y.o.; mean 35 y.o.) showing no abnormal findings on the pancreatogram, who had already undergone ERCP. In the Cambridge classification mild chronic pancreatitis is defined as "more than 3 abnormal side branches with a normal main pancreatic duct". In the present study, we selected patients in their 30's, because we wanted to exclude those with ductal changes associated with aging. In all the patients the maximum diameter of the main pancreatic duct was within 2 mm and the ultrasonogram and CT revealed no findings suggestive of chronic pancreatitis, such as atrophy, calcification, dilatation or stenosis of the pancreatic duct. The control subjects had no pancreatic diseases and were selected from a population that had undergone ERCP for a suspected biliary disease. Patients with mild chronic pancreatitis were selected from a population that had been subjected to ERCP for recurrent attacks of abdominal pain associated with increased serum levels of amylase and lipase in the absence of conclusive US or CT findings of chronic pancreatitis. The symptomatic period was between 15 and 54 mo. Secretin injection-MRCP was performed within 4 wk after ERCP. Informed consent was obtained from all patients after explanation of the examination process. The study protocol was approved by the ethical committee of our institution.

MRCP

After a 6-h fast, MR studies were performed on a 1.5 T scanner using a phased-array multi-coil. With the patients in the supine position, 100 U of secretin (Eisai Corporation, Tokyo, Japan) was injected intravenously. Half-Fourier fast echo sequences with the single slice method were obtained in the coronal plane and left and right oblique planes to emphasize the pancreatic body, head and tail, respectively. MRCP images of axial planes were also added on demand. The sequence parameters were: effective echo time, 250 ms; echo train length, 212; matrix, 384 × 384; FOV, 25 to 35 cm. A thin slice, thickness of 10-30 mm, was adopted to delineate the fine structure of the minimally dilated branches. The acquisition time for an image was 3 s with complete breath-holding. Acquisition was repeated every 30 s during 10 min.

Image analysis

Only clear MRCP images of the pancreatic ducts were regarded as acceptable. MRCP images were reviewed using ERCP as the gold standard. Two sets of MRCP films and one set of ERCP films were prepared so that each reviewer could work separately on the films. MRCP images were interpreted independently by two reviewers (Sai J and Takayashi Y) and ERCP images by another reviewer (Kubokawa Y); all of them were experienced in pancreaticobiliary imaging. Each MRCP and ERCP film was randomly numbered, and reviewers were blinded to the clinical information associated with each film. Abnormal branches of the pancreatic ducts were documented with markings on the duplicate films of ERCP and MRCP by each reviewer, who did not participate in the examination; visible branch pancreatic ducts were regarded as abnormal branch pancreatic ducts on MRCP images because they are not detectable on MRCP except in pathologic cases[10]. Then MRCP images were compared with the ERCP images for the number and location of the abnormal branch pancreatic ducts, lesion by lesion under consensus.

Statistical analysis

The sensitivity and specificity for the diagnosis of abnormal branch pancreatic ducts and mild chronic pancreatitis (Cambridge classification) determined by each reviewer was examined. Regarding the diagnosis of ectatic branch pancreatic duct and mild chronic pancreatitis on MRCP images, the interobserver agreement was analyzed with κstatistics: values were classified as excellent (above 0.75), fair to good (0.40 to 0.75), or poor (below 0.40).

RESULTS

Successful MRCP images were obtained after secretin injection in patients with mild chronic pancreatitis and control subjects. The rates for visualization of the main pancreatic duct on MRCP were 100%.

Abnormal branch ducts delineated on MRCP images correlated with ERCP images, lesion by lesion. The sensitivity and specificity for the diagnosis of abnormal branch pancreatic ducts determined by the two reviewers were 55%-63% and 75%-83% in the head, 57%-64% and 82%-83% in the body, and 44%-44% and 72%-76% in the tail of the pancreas, respectively (Table 1a, b). The sensitivity and specificity for the diagnosis of mild chronic pancreatitis (Cambridge classification) was 56%-63% and 92%-92%, respectively (Table 2). Interobserver agreement (κ statistics) for identifying abnormal branch pancreatic ducts and for diagnosing mild chronic pancreatitis was good to excellent (Table 3).

DISCUSSION

Early-stage chronic pancreatitis is difficult to diagnose because less obvious changes occur over time, before the development of overt exocrine or endocrine insufficiency and visible structural abnormalities[17]. Therefore, a major diagnostic challenge occurs when patients have clinical signs suggestive of chronic pancreatitis, in the absence of demonstrable pancreatic abnormalities on ultrasonography and CT. Reversely, in patients with advanced disease, findings such as atrophy, calcification, dilatation and stenosis of the pancreatic duct are obvious[18]. One of the earliest findings of chronic pancreatitis is an abnormal branch of the pancreatic duct detected by ERCP[2,18]. ERCP is the sensitive method for the diagnosis of early-stage chronic pancreatitis[19-21]; however, it is invasive and associated with complications[22,23], because sufficient contrast medium must be injected to the peripheral branches of the pancreatic ducts when mild changes of chronic pancreatitis are sought[24].

Today, diagnostic ERP is challenged by MRCP, which is a noninvasive alternative to diagnostic ERP for the evaluation of normal and diseased pancreatic ducts[5-9]. However, it is difficult to delineate minimally dilated

Table 1a Sensitivity and specificity of MRCP for abnormal branch pancreatic ducts as compared with ERCP by Reviewer 1. Result of the assessment of images

Location of abnormal branch pancreatic ducts	Number of lesions				Sensitivity (%)	Specificity (%)
	TP	TN	FP	FN		
Head	25	40	13	15	25/40 (63)	40/53 (75)
Body	16	88	18	12	16/28 (57)	88/106 (83)
Tail	4	19	6	5	4/9 (44)	19/25 (76)

TP: True positive; TN: True negative; FP: False positive; FN: False negative; Numbers are numbers of patients (numbers in parentheses are percentages).

Table 1b Sensitivity and specificity of MRCP for abnormal branch pancreatic ducts as compared with ERCP findings by Reviewer 2. Results of the assessment of images

Location of abnormal branch pancreatic ducts	Number of Lesions				Sensitivity (%)	Specificity (%)
	TP	TN	FP	FN		
Head	22	44	9	18	22/40 (55)	44/53 (83)
Body	18	87	19	10	18/28 (64)	87/106 (82)
Tail	4	18	7	5	4/9 (44)	18/25 (72)

branch pancreatic ducts on MRCP despite its improved resolution because while on ERCP images the collapsed branches and main pancreatic duct are distended by the contrast medium, MRCP reflects the physiologic state of the pancreatic duct.

Since Takehara *et al*[10] first reported the MRCP images of moderate to severe chronic pancreatitis obtained after the injection of secretin; several authors have reported the usefulness of secretin injection-MRCP in the diagnosis of chronic pancreatitis[12-15]. Secretin stimulates fluid secretion in the ductal system; and increases the tonus of the sphincter of Oddi during the first 5 min, inhibiting the release of fluid through the papilla of Vater[9]. Therefore, secretin increases the absolute volume of intraductal free water and fills the collapsed branches with fluid[10], facilitating the detection of mild ductal changes in patients with mild chronic pancreatitis, even when no abnormalities can be demonstrated by MRCP under physiologic conditions. According to Mansfredi *et al*[25], visualization of the pancreatic duct branches improved from 71% to 100% in 93 patients with severe chronic pancreatitis and from 4% to 63% in 84 patients suspected of having pancreatic disease. Since the pancreatic juice secretion decreases in patients with advanced chronic pancreatitis, secretin injection-MRCP could be of diagnostic value for those with early-stage chronic pancreatitis, although the occurrence of mild chronic pancreatitis was underestimated by secretin injection-MRCP compared with ERCP in the present study.

There were some limitations to the present study. MRCP should be interpreted by someone blinded to the clinical information to avoid biases. Furthermore, although ERCP was chosen as the gold standard, it is well known that chronic pancreatitis may exist in the presence of a normal pancreatogram. Besides, parenchymal abnormalities may precede ductal and functional changes, and EUS is considered a more sensitive test for parenchymal abnormalities[26-30]. Therefore, the diagnostic value of secretin injection-MRCP for mild chronic pancreatitis might be limited.

Table 2 Sensitivity and specificity of MRCP for mild chronic pancreatitis (Cambridge Classification)

	Sensitivity (%)	Specificity (%)
Reviewer 1	10/16 (63)	9/12 (75)
Reviewer 2	9/16 (56)	10/12 (83)

Table 3 Interobserver agreement regarding the identification of abnormal branch pancreatic ducts (κ statics)

Location of abnormal branch pancreatic ducts	κ value
Abnormal branch, head	0.67
Abnormal branch, body	0.68
Abnormal branch, tail	0.86
Diagnosis of mild chronic pancreatitis	0.79

In conclusion, secretin injection-MRCP might allow an earlier diagnosis of chronic pancreatitis and reduce the rate of false-negative cases detected with MRCP.

COMMENTS

Background

Early-stage chronic pancreatitis is difficult to diagnose because less obvious changes occur over time. One of the earliest findings of chronic pancreatitis is an abnormal branch of the pancreatic duct detected by endoscopic retrograde cholangiopancreatography (ERCP). On the other hand, MR cholangiopancreatography (MRCP) is expected to become valuable alternative to ERCP. However, diagnosis of mild ductal changes of chronic pancreatitis using magnetic resonance cholangiopancreatography (MRCP) is quite challenging, because visualization of the branch pancreatic ducts is not good.

Research frontiers

We examined the diagnostic accuracy of secretin injection-MRCP regarding abnormal branch pancreatic ducts associated with mild chronic pancreatitis (Cambridge classification) using ERCP for comparison, and there were no other related or similar studies.

Innovation and breakthrough

The sensitivity and specificity for the diagnosis of mild chronic pancreatitis (Cambridge classification) was 56%-63% and 92%-92%, respectively. Secretin injection-MRCP might allow an early diagnosis of chronic pancreatitis.

Application

The application of this study could be expanded to diagnose early chronic pancreatitis in larger series.

Terminology

Secretin injection-MRCP was acquired as follows: with the patients in the supine position, 100 U of secretin was injected intravenously. MRCP was repeated every 30 s during 10 min.

Peer review

Secretin injection-MRCP could be a useful method for the diagnosis of mild chronic pancreatitis (Cambridge Classification), although the occurrence was underestimated compared with ERCP.

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RAPID COMMUNICATION

Ameliorative effects of bombesin and neurotensin on trinitrobenzene sulphonic acid-induced colitis, oxidative damage and apoptosis in rats

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Abstract

AIM: To investigate the effects of bombesin (BBS) and neurotensin (NTS) on apoptosis and colitis in an ulcerative colitis model.

METHODS: In this study, a total of 50 rats were divided equally into 5 groups. In the control group, no colitis induction or drug administration was performed. Colitis was induced in all other groups. Following the induction of colitis, BBS, NTS or both were applied to three groups of rats. The remaining group (colitis group) received no treatment. On the 11th d after induction of colitis and drug treatment, blood samples were collected for TNF-α and IL-6 level studies. Malondialdehyde (MDA), carbonyl, myeloperoxidase (MPO) and caspase-3 activities, as well as histopathological findings, evaluated in colonic tissues.

RESULTS: According to the macroscopic and microscopic findings, the study groups treated with BBS, NTS and BBS + NTS showed significantly lower damage and inflammation compared with the colitis group (macroscopic score, 2.1 ± 0.87, 3.7 ± 0.94 and 2.1 ± 0.87 *vs* 7.3 ± 0.94; microscopic score, 2.0 ± 0.66, 3.3 ± 0.82 and 1.8 ± 0.63 *vs* 5.2 ± 0.78, $P < 0.01$). TNF-α and IL-6 levels were increased significantly in all groups compared with the control group. These increases were significantly smaller in the BBS, NTS and BBS + NTS groups compared with the colitis group (TNF-α levels, 169.69 ± 53.56, 245.86 ± 64.85 and 175.54 ± 42.19 *vs* 556.44 ± 49.82; IL-6 levels, 443.30 ± 53.99, 612.80 ± 70.39 and 396.80 ± 78.43 *vs* 1505.90 ± 222.23, $P < 0.05$). The colonic MPO and MDA levels were significantly lower in control, BBS, NTS and BBS + NTS groups than in the colitis group (MPO levels, 24.36 ± 8.10, 40.51 ± 8.67 and 25.83 ± 6.43 *vs* 161.47 ± 38.24; MDA levels, 4.70 ± 1.41, 6.55 ± 1.12 and 4.51 ± 0.54 *vs* 15.60 ± 1.88, $P < 0.05$). Carbonyl content and caspase-3 levels were higher in the colitis and NTS groups than in control, BBS and BBS + NTS groups (carbonyl levels, 553.99 ± 59.58 and 336.26 ± 35.72 *vs* 209.76 ± 30.92, 219.76 ± 25.77 and 220.34 ± 36.95; caspase-3 levels, 451.70 ± 68.27 and 216.20 ± 28.17 *vs* 28.60 ± 6.46, 170.50 ± 32.37 and 166.50 ± 30.95, $P < 0.05$).

CONCLUSION: The results of this study suggest BBS and NTS, through their anti-inflammatory actions, support the maintenance of colonic integrity and merit consideration as potential agents for ameliorating colonic inflammation.

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Key words: Bombesin; Neurotensin; Colitis; Apoptosis

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INTRODUCTION

Inflammatory bowel diseases (IBDs), including Crohn's

disease (CD) and ulcerative colitis (UC), are chronic relapsing inflammatory conditions of unknown etiology. The most common symptoms of both UC and CD are diarrhea, which may be profuse and bloody, and abdominal pain[1].

The exact pathogenesis in IBD is poorly understood, but evidence suggests IBD involves interactions between the immune system, genetic susceptibility and the environment[1]. Some studies have analyzed the role of a variety of soluble peptides and other mediators that, once secreted in a co-ordinated fashion in the injured area, are able to restore mucosal integrity[2].

CD affects the full thickness of the wall of the colon or the small intestine, whereas UC primarily affects the mucosa of the colon. Histologically, the affected tissue shows ulceration and the infiltration of inflammatory cells from the circulation[3].

Neuropeptides are small molecules that, in the intestine, modulate several biological processes and modify the activities of cells responsible either for triggering tissue damage or promoting healing[4,5]. The tetradecapeptide bombesin (BBS) was originally isolated from the skin of the amphibian *Bombina bombina*, and is analogous to mammalian gastrin-releasing peptide[6]. In the gastrointestinal tract, BBS stimulates the secretion of various gut hormones and peptides (for example, gastrin, somatostatin, cholecystokinine, insulin, glucagon, motilin, pancreatic polypeptide and neurotensin), exerts trophic effects on intestinal mucosa and pancreas, and stimulates intestinal motility[7,8]. It has been shown BBS improves intestinal integrity in experimental models of gut barrier dysfunction, like administration of methotrexate and elemental diets[9,10], and that it also has mitogenic effects[11]. Neurotensin (NTS) is a tridecapeptide, found mainly in the brain and gut. Intestinal NTS is produced by a discrete population of endocrine cells scattered throughout the jejeuno-ileal mucosa[12]. The peptide is released into the circulation after food ingestion. Its digestive functions include stimulation of pancreaticobiliary secretions, intestinal blood flow, inhibition of gastric acid secretion and motility, stimulation of colon motility and inhibition of jejuno-ileum motility. It is a potent trophic agent for the small and large intestines[13,14]. NTS has been shown to prevent intestinal atrophy induced by feeding rats an elemental diet, and to enhance intestinal mucosal restoration after small bowel resection[15]; it also has immunoregulatory functions[16].

The intact mucosa and an indigenous intestinal flora are important components of body defenses against luminal pathogenic bacteria. The epithelia of the colon are dynamic and rapidly proliferating tissues that are profoundly affected by changes in luminal contents and injury. The aim of this study was to determine the effects of BBS and NTS on an experimental colitis model, because of their potent trophic, metabolic and mitogenic effects.

MATERIALS AND METHODS

Animals

Male Wistar-albino rats (Erciyes University Hakan Cetinsaya Experimental and Clinical Research Center, Kayseri, Turkey) weighing 225-290 g were fed ad libitum a nutritionally balanced rodent diet and water. They were kept under constant environmental conditions. Water only was provided during the 12 h preceding the experiments. Our institutional ethical committee approved the experimental procedures of this study.

Experimental design

Fifty Wistar-albino rats (28-32 wk) were used in the study. The rats were divided into 5 groups containing 10 rats each: Group I (Control) = normal animals; Group Ⅱ (Colitis) = induction of experimental colitis without further treatment; Group Ⅲ (BBS) = induction of experimental colitis plus administration of bombesin; Group Ⅳ (NTS) = induction of experimental colitis plus administration of neurotensin; and Group Ⅴ (BBS + NTS) = induction of experimental colitis plus administration of bombesin and neurotensin together.

Experimental procedures

Colitis was induced as previously described[17]. Briefly, rats were lightly anesthetized with halothane, and an infant feeding tube (Unoplast A/S DK 330; Hundested, Denmark) fitted onto a blunt 18-gauge needle was inserted rectally. The tip of the tube was placed nearly 8 cm into the colon and 25 mg 2,4,6- trinitrobenzene sulphonic acid (Sigma, St. Louis, Missouri) mixed with 0.25 mL 30% ethanol (TNBS-E) was intracolonically instilled. After instillation, the rats were supported in the supine position until recovery from anesthesia to prevent immediate anal leakage of the instillate. The control group rats received intracolonic saline. For the 10 subsequent days, the animals in groups 3 and 5 were treated daily with BBS (10 μg/kg, subcutaneously, 3 times a day), and those in groups 4 and 5 were treated daily with NTS (300 μg/kg, intraperitoneally, once a day). Rats in control and colitis groups received sterile saline at the same volumes and same times. All injections were given after topical application of an antiseptic solution of povidone iodine 10%.

All rats were fed standard rat chow for 10 d. On the 11th d, all animals were operated on. Rats were anesthetized by intraperitoneal injection of ketamine-HCl (10 mg/kg; Ketalar®, Eczacibasi, Istanbul, Turkey) and xylazin (3 mg/kg; Rompun® 2%, Bayer, Germany). The abdominal skin was cleansed with povidone-iodine after shaving. All procedures were performed under sterile conditions by one surgeon. The abdomen was opened by a midline incision, and a 2-mL blood sample was collected from the portal vein to measure the levels of TNF-α and IL-6. The distal colon was removed for analysis of myeloperoxidase (MPO) activity, carbonyl content (protein oxidation), lipid peroxidation (malondialdehyde [MDA]), caspase-3 activity and histologic examination. At the end of surgical procedure, all rats were sacrificed by overdose ether inhalation.

Peptides preparation

Stock solutions of BBS (Sigma Chemical Co) and NTS (Sigma Chemical Co) were prepared by first dissolving the amount of peptide needed for the study in 1 mL of sterile

Table 1 Colon macroscopic scoring system

Score	Criteria
0	No damage
1	Hyperemia
2	Hyperemia and thickening without ulceration
3	Ulceration at a single site
4	Two or more sites of ulceration or inflammation
5	Ulceration or inflammation extending > 1 cm along the length of colon
6-10	Damage covering > 2 cm along the lenght of colon, with the score being increased by 1 for each additional centimeter of involvement

water containing 0.1% (weight/volume) bovine serum albumin, and then diluted with normal saline containing 1% bovine serum albumin, so that the amounts of BBS and NTS needed for each injection were contained in a volume of 0.1 mL. Aliquots of 1% bovine serum albumin and BBS were stored in plastic tubes at -20℃. To prolong the rate of absorption after each injection, BBS in saline was mixed 1:4 (volume/volume ratio) with 8% (weight/volume) hydrolyzed gelatin (Sigma Chemical Co) before administration. A final volume of 0.5 mL containing 10 μg BBS/kg body weight, was injected subcutaneously 3 times daily[7]. The NTS solution was divided into equal aliquots of 0.1 mL that were stored in glass vials at -20℃. At the time of administration, each aliquot was further diluted with 0.4 mL of sterile saline to a final volume of 0.5 mL, which was given intraperitoneally as a bolus injection containing 300 μg NTS/kg body weight[18].

Determination of serum tumor necrosis factor-alpha and interleukin-6 levels

The levels of serum tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were determined using specific enzyme-linked immunosorbent assay kits (BioSource Rat TNF-α ELISA and BioSource Rat IL-6 ELISA). The results were expressed as pg/mL.

Assessment of colonic damage

The distal colon was removed, opened longitudinally and fecal material was rinsed with a gentle spray of 0.9% saline. The freshly opened colonic segments were pinned out on a wax block and examined by a pathologist who was blinded to the treatment.

Colon macroscopic score

The extent of mucosal damage was assessed using the colon macroscopic scoring system of Wallace *et al* (Table 1)[19].

Colon microscopic score

Tissue samles were fixed with 10% formaldehyde solution for 24 h and transferred to 70% ethanol solution. The tissues were embedded in parafin; 4-μm thick sections were made, and histologic examination was performed under a light microscope after hematoxylin and eosin staining. A pathologist who was unaware of the animals' groupings evaluated the specimens. Tissues were assessed for the presence and activity of colitis as well as the extent of tissue damage using a large number of serial sections. Colonic inflammation was assessed using a modification of the histopathologic grading system of MacPherson and Pfeifer (Table 2)[20].

Table 2 Histopathologic grading scale of TNBS-E-induced colitis

Grade of colitis	Microscopic findings
0	No damage
Ⅰ	Mild mocosal and/or submucosal inflammatory infiltrate and edema: punctuate mucosal erosions often associated with capillary proliferation, muscularis mucosa intact
Ⅱ	Grade Ⅰ changes involving 50% of the specimen
Ⅲ	Prominent inflammatory infiltrate and edema frequently with deeper areas of ulceration extending through the muscularis mucosa into the submucosa, rare inflammatory cells invading the muscularis propria but without muscle necrosis
Ⅳ	Grade Ⅲ changes involving 50% of the specimen
Ⅴ	Extensive ulceration with coagulative necrosis bordered inferiorly by numerous neutrophils and fewer mononuclear cells, necrosis extends deeply into the muscularis propria
Ⅵ	Grade Ⅴ changes involving 50% of the specimen

Myeloperoxidase measurement in colon tissue

After being scored, a sample of distal colon was frozen for subsequent measurement of MPO activity as an index of granulocyte infiltration[19]. The method described by Bradley *et al* was used to measure MPO activity in colon homogenates[21]. The previously frozen colon tissues were homogenized for 30 s in 4 mL of 20 mmol/L potassium phosphate buffer, pH 7.4, and centrifuged for 30 min at 40 000 × *g* at 4℃. The pellet was resuspended in 4 mL of 50-mmol/L potassium phosphate buffer, pH 6.0, containing 0.5 g/dL hexadecyltrimethyl ammonium bromide. Samples were sonicated for 90 s at full power, incubated in a 60℃ water bath for 2 h and centrifuged. The supernatant (0.1 mL) was added to 2.9 mL of 50-mmol/L potassium phosphate buffer, pH 6.0, containing 0.167 mg/mL dianisidine and 0.0005% hydrogen peroxide. Absorbance of 460 nm visible light (A460) was measured for 3 min. The results were expressed as units of enzyme activity per gram of wet tissue weight (U/g tissue).

Malondialdehyde measurement in colon tissue

One part of the tissue was homogenized in 10 parts of 15 mmol/L KCl for the MDA assay. MDA, which is the end product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. Measurement of MDA content by TBA reactivity is the most widely used methods to assess lipid peroxidation[22]. The principle of the method is based on measurement of the absorbance of the pink color produced by the interaction of TBA with MDA at 530 nm. Values were expressed as nmol/g wet tissue weight.

Determination of carbonyl content

Tissue carbonyl molecule formation was determined to reflect protein oxidation as a biochemical marker of oxidative damage owing to colitis. Protein oxidation

Table 3 Comparison of macroscopic and microscopic colonic damage scores

Colonic damage score		Group Ⅰ Control	Group Ⅱ Colitis	Group Ⅲ BBS	Group Ⅳ NTS	Group Ⅴ BBS and NTS	*P*
Macroscopic score	mean ± SD	0.3 ± 0.48	7.3 ± 0.94	2.1 ± 0.87	3.7 ± 0.94	2.1 ± 0.87	< 0.01
	Median (Min-Max)	0 (0.0-1.0)[b]	7 (6.0-9.0)[d]	2 (1.0-3.0)[b]	4 (2.0-5.0)[f]	2 (1.0-3.0)[b]	
Microscopic score	mean ± SD	0.4 ± 0.69	5.2 ± 0.78	2.0 ± 0.66	3.3 ± 0.82	1.8 ± 0.63	< 0.01
	Median (Min-Max)	0 (0.0-2.0)[b]	5 (4.0-6.0)[d]	2 (1.0-3.0)[b]	3.5 (2.0-4.0)[f]	2 (1.0-3.0)[b]	

Data are expressed as means ± SD and median (min-max). [b]$P < 0.01$ *vs* colitis and NTS; [d]$P < 0.01$ *vs* BBS, NTS and BBS and NTS; [f]$P < 0.01$ *vs* colitis, control, BBS and BBS and NTS. BBS: Bombesin; NTS: Neurotensin.

was assayed by a modified streptomycin sulfate, 2,4-dinitrophenylhydrazine-based method, and expressed in nmol total carbonyl groups/mg total protein[23].

Caspase-3 proteolytic activity

The caspase-3 activity ratio was calculated using a colorimetric assay (Chemicon International, Temecula, CA). Cells harvested from the different treatment groups were lysed and 200 μg of protein was tested for protease activity by the addition of a caspase-specific substrate peptide, DVED-pnitroaniline. Caspase-3 cleavage of the peptide releases the chromophore p-nitroaniline (p-NA), which was quantitated spectro-photometrically at 405 nm. The level of caspase-3 enzymatic activity in the cell lysate was directly proportional to the extent of the color reaction. The results are expressed as the fold increase of caspase activity in apoptotic cells relative to their respective controls. In background reactions, no DVED-p-NA substrate was added, and the values obtained were subtracted from experimental results before calculating the fold increase[24].

Statistical analysis

The data are presented as means ± SD. One-way analysis of variance (ANOVA) was used to compare the levels of serum TNF-α, IL-6, colon MDA and MPO, and caspase-3, as well as carbonyl content, among the 5 groups. The Tamhane test was used as a post hoc test for multiple comparisons between groups. Non-parametric Kruskal-Wallis tests were used to compare macroscopic and microscopic damage scores. Dunn's test was used for multiple comparisons between groups. Data were analyzed using Statistical Package for the Social Sciences (SPSS) for Windows (version 13.0). Significance was set at $P < 0.05$.

RESULTS

Macroscopic colonic damage

Table 3 shows the results of assessment of gross colonic injury. In rats treated with BBS in which a macroscopic response was evident, there was minimal damage to the surface epithelium, and there was mild inflammation of the mucosa, but no transmural inflammation. In the colitis group, the damage comprised broad mucosal ulcers with a surface layer of necrotic slough, accumulation of mesenteric fat and fibrinous adhesions to the bowel. Macroscopic colonic damage score was highest in the colitis group (median; 7.0). This score was significantly lower in BBS and BBS + NTS groups, but higher than that in control rats ($P < 0.01$). Although the score in rats in NTS group was higher than those in rats in the BBS and BBS + NTS, and lower than that in the colitis group, there was no significant difference ($P > 0.05$) (Table 3).

Microscopic colonic damage

Histopathologic evaluation of the control group was within normal limits. Microscopic examination showed low levels of damage or inflammation in rats treated with BBS (groups Ⅲ and Ⅴ), whereas in rats with colitis, there was widespread destruction of the mucosa with transmural infiltration of neutrophils, monocytes and lymphocytes ($P < 0.01$) (Table 3).

Serum tumor necrosis factor-alpha and interleukin-6 levels

Serum TNF-α and IL-6 levels in the colitis and study groups were significantly increased at d 11 compared with the control group ($P < 0.05$). This increase was significantly smaller in the BBS, NTS and BBS + NTS groups than in the colitis group ($P < 0.05$) (Figures 1 and 2).

Myeloperoxidase levels

The colonic tissue MPO levels were higher in the colitis group than in the control, BBS, NTS and BBS + NTS groups ($P < 0.001$). There were no significant differences between the control and BBS groups ($P = 0.27$), or between the BBS and BBS + NTS groups ($P = 1.00$), whereas the MPO levels in the control and BBS + NTS groups were statistically significant different ($P = 0.001$). The increase in the NTS group was significantly high than the increase in the BBS ($P = 0.004$) and BBS + NTS groups ($P = 0.005$) (Figure 3).

Malondialdehyde levels

MDA levels were highest in the colitis group, and were significantly higher in the BBS, NTS and BBS + NTS groups compared with the control group ($P < 0.01$). There was no statistically significant difference between BBS and BBS + NTS groups ($P = 1.00$), but the MDA levels in these two groups were significantly higher than those in the NTS group ($P = 0.047$ and $P = 0.002$) (Figure 4).

Carbonyl contents

Carbonyl contents were higher in the colitis and NTS groups compared with the control, BBS and BBS + NTS groups ($P < 0.001$). The carbonyl contents of the control, BBS and BBS + NTS groups were not significantly different ($P > 0.05$, range; 0.997-1.00). The carbonyl content of the NTS group was significantly higher than

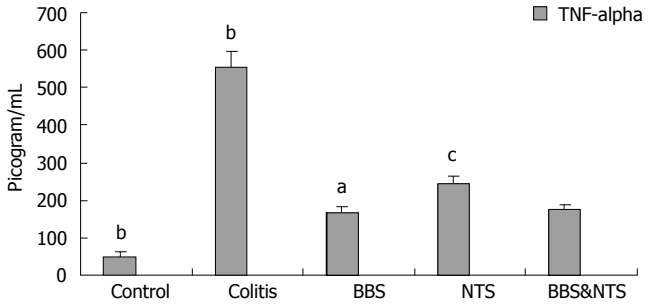


Figure 1 Serum tumor necrosis factor-alpha (TNF-alpha) concentrations in rats. Vertical columns and error bars represent mean ± SD. $^{b}P < 0.001$ *vs* BBS, NTS and BBS and NTS; $^{a}P < 0.05$ *vs* control, colitis and NTS; $^{c}P < 0.05$ *vs* control, colitis, BBS and BBS and NTS. BBS: Bombesin; NTS: Neurotensin.

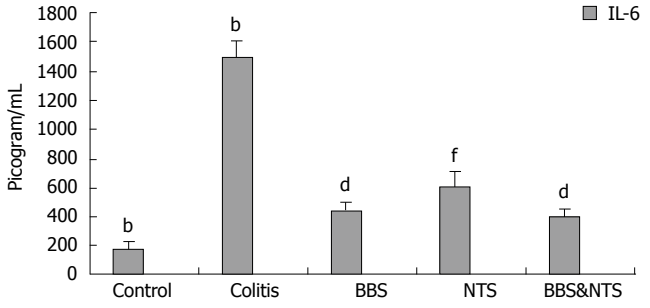


Figure 2 Serum interleukin-6 (IL-6) concentrations in rats. Vertical columns and error bars represent mean ± SD. $^{b}P < 0.001$ *vs* BBS, NTS and BBS&NTS; $^{d}P < 0.001$ *vs* control, colitis and NTS; $^{f}P < 0.001$ *vs* control, colitis and BBS and BBS and NTS. BBS: Bombesin; NTS: Neurotensin.

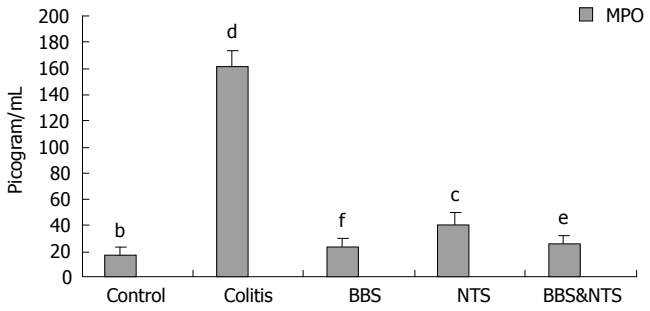


Figure 3 Colon myeloperoxidase (MPO) concentrations in rats. Vertical columns and error bars represent mean ± SD. $^{b}P < 0.001$ *vs* colitis and NTS; $^{d}P < 0.001$ *vs* control, BBS, NTS and BBS and NTS; $^{f}P < 0.001$ *vs* colitis and NTS; $^{c}P < 0.05$ *vs* control, colitis, BBS and BBS and NTS; $^{e}P < 0.05$ *vs* control, colitis, BBS and NTS. BBS: Bombesin; NTS: Neurotensin.

that of the control, BBS and BBS + NTS groups, but lower than that in the colitis group ($P < 0.001$) (Figure 5).

Caspase-3 levels

Colon caspase levels were higher in the colitis and NTS groups than in the control, BBS and BBS + NTS groups ($P < 0.05$, range; 0.001-0.034). There was no statistically significant difference between the BBS and BBS + NTS groups ($P = 1.00$), but the increases in caspase-3 levels in these two groups were significantly smaller than those in the control, colitis and NTS groups ($P < 0.05$, range; 0.001-0.034) (Figure 6).

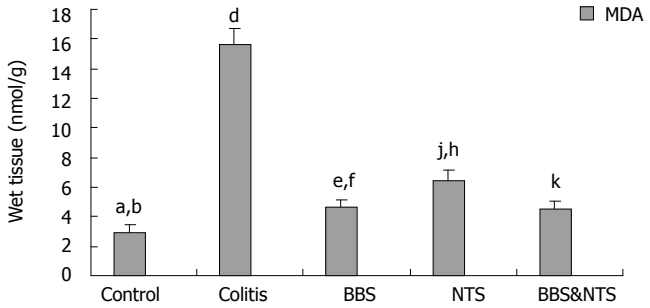


Figure 4 Colon malondialdehyde (MDA) concentrations in rats. Vertical columns and error bars represent mean ± SD. $^{b}P < 0.001$ *vs* colitis, NTS and BBS&NTS and $^{a}P < 0.05$ *vs* BBS; $^{d}P < 0.001$ *vs* control, BBS, NTS and BBS&NTS; $^{f}P < 0.001$ *vs* colitis and $^{e}P < 0.05$ *vs* control and NTS; $^{h}P < 0.001$ *vs* control and colitis, $^{j}P < 0.05$ *vs* BBS and BBS and NTS; $^{k}P < 0.001$ *vs* control, colitis and NTS. BBS: Bombesin; NTS: Neurotensin.

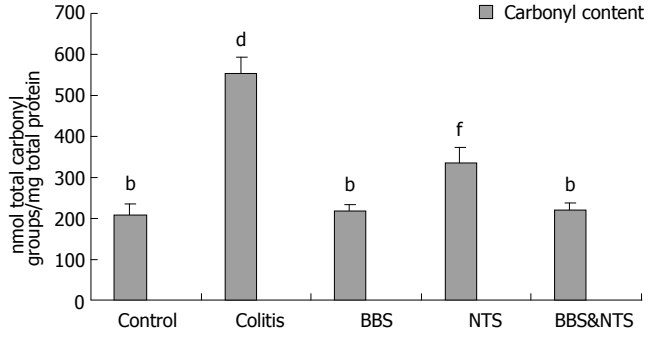


Figure 5 Colonic carbonyl content in rats. Vertical columns and error bars represent mean ± SD. $^{b}P < 0.001$ *vs* colitis and NTS; $^{d}P < 0.001$ *vs* control, BBS, NTS and BBS&NTS; $^{f}P < 0.001$ *vs* control, colitis, BBS and BBS&NTS; BBS: Bombesin; NTS: Neurotensin.

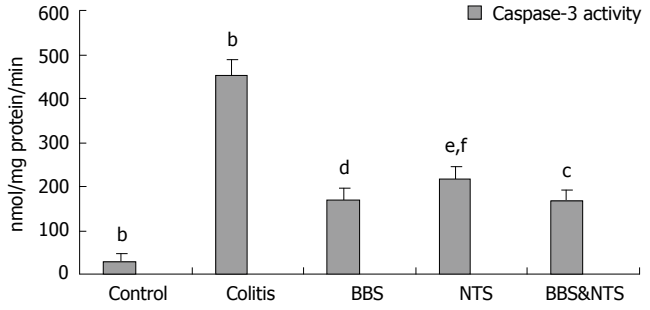


Figure 6 Colonic caspase-3 activity of all groups. Vertical columns and error bars represent mean ± SD. $^{b}P < 0.001$ *vs* BBS, NTS and BBS&NTS; $^{d}P < 0.001$ *vs* control, colitis and $^{c}P < 0.05$ *vs* NTS; $^{f}P < 0.001$ *vs* control and colitis, $^{e}P < 0.05$ *vs* BBS and BBS and NTS; BBS: Bombesin; NTS: Neurotensin.

DISCUSSION

IBDs are chronic intestinal inflammatory disorders that are considered to be the result of an inappropriate response of the immune system to luminal antigens[25]. Studies over the past several years have shown a variety of soluble peptides, prostaglandins, growth factors, cytokines and chemokines are secreted in a coordinated fashion in the injured area to restore mucosal integrity[26-28]. Although experimental animal models of colitis are of value in eliciting some of the mechanisms underlying IBDs, different models

differ in their relative use. Most animal models lack certain characteristics of IBDs and model acute events rather than the chronic features typical of IBDs. TNBS colitis has been well characterized and has clinical, biochemical and pathologic similarities to colonic CD. Animals with TNBS model colitis respond to drugs that have proven useful in IBDs[19]. Therefore, we used TNBS-induced colitis, which has the dual advantage of chronicity and acuteness so often seen in patients with IBD. Some other advantages of this model of colitis are its reasonable reproducibility and its value in assessing the efficacy of therapeutic agents commonly used for the treatment of colitis[19].

BBS and NTS are biologically active neuropeptides that are widely distributed in the central and peripheral nervous systems as well as in the endocrine systems. They have multiple biological effects, such as gastrointestinal motility, mucosal blood flow, cell growth and immunoregulation[29]. Their biological effects may result from direct actions of BBS and NTS, as neurotransmitters, on the target organ, or indirectly through the action of gastrointestinal hormones released in response to these neuropeptides. Furthermore, the nature and amplitude of neuropeptide-mediated effects may be different in normal tissue and in inflammatory disorders, because the levels of receptors and cell populations expressing neuropeptide receptors may be strikingly different[30]. In this study, we aimed to assess the role of BBS and NTS in a model of colitis using TNBS, a well-accepted model for IBD.

Although BBS and NTS improve intestinal barrier function in an experimental colitis model[7,14,18], we don't know the precise mechanisms underlying these effects. Therefore, we aimed to evaluate the effects of BBS and NTS on lipid peroxidation, protein oxidation and apoptosis in a colitis model. We also aimed to analyse the molecular mechanisms of these effects.

In the colitis group, increased macroscopic and microscopic damage scores revealed severe inflammatory changes. BBS and NTS treatment attenuated the colonic damage, suggesting these agents may have an additional protective role supporting colonic integrity. Although both agents significantly reduced macroscopic and microscopic colonic damage scores, we don't know if the effects of these two agents are additive or synergistic, and whether these effects are dose-dependent or not. There is no report in the literature about the therapeutic doses of BBS and NTS in experimental colitis models. However, in most studies, both agents were administered at the same doses[7,31]. Brun *et al* reported the mucosal level of NTS showed a 5.8-fold increase in an experimental colitis model compared with normal mice[4]. On the contrary, in patients with chronic inflammatory disorders, neuropeptide levels have been reported to be low[32]. All of these observations suggest the neuropeptide response depends on the severity of the inflammatory process and the site of the event. Receptor changes may cause these effects. Of the three mammalian bombesin-like receptors, only the type-2 receptor is present in the human intestine. The receptor types present and their localization were not altered in patients with IBD, although there was an obvious decrease in the number of binding sites in the smooth muscle of patients with IBD compared with normal control patients. In the mucosa of patients with IBD, very few binding sites for BBS were seen[33]. This suggests that if the administration of BBS has any effect, it will be observed in the human mucosa, acting via indirect mechanisms such as release of other trophic agents, for example, cholecystokinin, neurotensin.

TNF-α and IL-6 are multifunctional cytokines produced primarily by activated monocytes and macrophages; they play a crucial role in the initiation and continuation of mucosal inflammation and immunity[34,35]. These cytokines are involved in many cell processes including apoptotic cell death, metabolism, inflammation, thrombosis and fibrinolysis[36]. TNF-α and IL-6 induce the production of other cytokines including adhesion molecules and arachidonic acid metabolites, and activation of immune and non-immune cells[37]. Systemic inhibition of soluble TNF-α by infliximab (chimeric anti- TNF-α mAbs) has been reported to induce remission in up to 50% of patients with CD and to significantly improve clinical symptoms in most patients[38]. Worledge *et al* reported that the administration of TNF-α antibodies effectively treated experimental colitis in rats[39]. Analysis of endoscopic biopsy samples from IBD patients revealed the involved colonic mucosa from patients with active disease contains larger amounts of IL-6 than colonic mucosa from patients in remission[40]. In another study, the administration of anti-IL-6 antibody was shown to ameliorate disease[41]. In our study, TNF-α and IL-6 levels were correlated with the increased level of inflammation in the colitis group. By contrast, TNF-α and IL-6 levels decreased in all study groups, this decrease was more significant in the BBS and BBS + NTS groups. At this point, TNF-α and IL-6 may be promising markers in monitoring the progress of IBD and the response to treatment. Such findings indicate blockade of TNF-α and IL-6 signaling are clinically advantageous in the treatment of IBDs. The results of our study suggest the therapeutic potential of BBS and NTS in human IBDs with decreases TNF-α and IL-6 levels. Assimakopoulos *et al* reported experimental obstructive jaundice induces regional loss of occludin expression in the intestinal epithelium, which may be a key contributing factor to the disruption of the mucosal barrier. The gut regulatory peptides BBS and NTS prevent this alteration leading to lower portal and systemic endotoxemia and inflammation[31]. These inflammatory responses are characterized by the release of cytokines and other proinflammatory mediators, such as TNF-α, IL-6 and oxygen-free radicals. These agents may produce injurious effects on the structure and function of tight junctions, compromising intestinal epithelial barrier function[35,37,42]. Specifically, it has been demonstrated TNF-α downregulates the human occludin promoter[43]. In addition, endotoxemia and inflammation reduce splanchnic blood flow and disrupt intestinal microcirculation, resulting in hypoxia of enterocytes and energy depletion[44]. Studies in epithelial cells have shown that adenosine triphosphate (ATP) depletion induces the structural perturbation of the tight junction[45]. Increased ATP levels in treatment groups may affect energy balance positively, with improved functional and morphological restoration.

Cell death by apoptosis plays an important role

in the regulation of intestinal epithelial homeostasis. Administration of anti-IL-6 and TNF-α antibodies to rats with different forms of experimental colitis leads to decreases in the severity of colitis and the occurrence of apoptotic cells in the inflammed tissues[46]. However, Atreya *et al* have reported the mechanisms underlying the therapeutic effects of anti-IL-6 antibodies are more profound; they affect the survival of $CD4^+$ T cells mediating inflammation such that the main action of the antibodies is on the cells inducing or producing the cytokines[47]. The potential relevance of modulating T-cell resistance against apoptosis as a therapeutic target in IBD is underlined by Zhou *et al*, who showed drugs that facilitate apoptosis can be used for the supression of T-cell mediated autoimmune disease[48]. Control animals showed basal levels of apoptotic activity, known as spontaneous apoptosis. TNBS-E-induced colitis resulted in a significant increase in the levels of proinflammatory cytokines and apoptosis, while BBS and NTS reduced the levels of proinflammatory cytokines and the rate of apoptotic death. Potential mechanism for increased apoptosis after TNBS-E induced colitis may implicate the excessive release of IL-6 and TNF-α, activation of cytotoxic T cells. Activated cytotoxic T cells induce death of inflammatory $CD4^+$ T cells via independent and possibly synergistic mechanisms. Assimakopoulos *et al* reported BBS and NTS signficantly decreased apoptosis in obstructed jaundiced rats, and they speculated this effect occurred by decreasing intestinal oxidative stress, which is an important apoptosis inducer[49]. In addition, it should be taken into consideration that neuropeptide-mediated effects may be modulated by complex interactions with other mediators involved in tissue restitution, such as chemokines[28]. An indirect mechanism may be related to a further reduction of intestinal lipid peroxidation, because oxidative state is involved in the modulation of cell proliferation and death[50]. The anti-apoptotic effect of BBS and NTS may be mediated by a reduction in endotoxemia and amelioration of the subsequent systemic inflammation, which may activate the apoptotic process. Another explanation of their anti-apoptotic action may be through induction of vasodilation[51], which improves intestinal microcirculation, thus preventing hypoxia, energy depletion and activation of apoptotic pathways. The anti-apoptotic effects of BBS and NTS may be mediated by acting on one or more factors. For this reason, the future of therapy for many types of IBDs might lie in combined anti-cytokine, anti-oxidant and anti-apoptotic therapies, rather than treatment with a single agent alone.

It has been proposed that oxygen-derived free radicals are implicated in the pathogenesis of IBDs[52]. Numerous studies showed MDA is formed in increased amounts in intestinal tissue[52,53]. MDA (lipid peroxidation) and carbonyls in cellular proteins (protein oxidation), generated by oxygen free radicals, is believed to be an important cause of damage to cell membranes[23,54]. Previous experimental studies had shown BBS and NTS improved gut barrier functions after chemically induced colitis[13,15]. Similarly, in our study, TNBS-E-induced colitis resulted in increased MDA and carbonyl production in colonic tissue. Additionally, BBS and NTS administration significantly reduced intestinal MDA and carbonyl content in rats with TNBS-E-induced colitis. These results provide evidence of an antioxidant action of BBS and NTS in the intestine in an experimental colitis model. These effects might be related to a reduction in endotoxemia or improvement in intestinal microcirculation induced by BBS and NTS, leading to oxidative metabolism. This antioxidant effect may be attributed to a direct receptor-mediated mechanism. Binding of neuropeptides to their specific receptors is followed by activation of protein kinase[55], which is known to activate intracellular antioxidant enzyme systems. An alternative explanation might be the reduction of endotoxemia, which is associated with the generation of oxygen free radicals *via* xanthine oxidase-dependent pathways. Moreover, improvement in intestinal microcirculation induced by regulatory neuropeptides prevents hypoxia and energy depletion, leading to oxidative metabolism. Since structural and functional integrity of mitochondria is crucial for the maintenance of cell metabolic homeostasis, this mechanism enhances cellular defenses against oxidant injury.

In our experiments, the inflammatory response caused by TNBS-E is described by an important increase in neutrophil infiltration into the colonic tissue, and the subsequent augmentation of MPO levels. This increase in MPO activity was substantially reduced in rats treated with BBS and NTS. Neutrophils play a crucial role in this regard by producing superoxide anions and a cascade of various reactive species. Activated neutrophils pass out of the circulation and enter the inflammed mucosa and submucosa of the colon during acute inflammation, leading to a large amount of production of reactive oxygen and nitrogen species that can contribute to intestinal injury[56]. MPO catalyzes the formation of such potent cytotoxic oxidants. The reduction in colonic MPO activity, as well as the histological findings of the absence of cellular infiltration following treatment with BBS and NTS, may indicate their antioxidant and anti-inflammatory effects.

The present study has shown TNBS-E-induced ulcerative colitis was associated with macroscopic, microscopic and biochemical changes. Medical treatment of IBDs is principally aimed at inhibiting the production of inflammatory mediators and at modulating the immune system. Treatment with BBS and NTS attenuated colitis as shown by the lower serum levels of TNF-α and IL-6, and the reduced colonic tissue levels of MPO, MDA, carbonyls and caspases. Additionally, these neuropeptides stimulate growth and regeneration of the intestinal mucosa. For these reasons, they have been implicated in the treatment of IBDs. However, further studies are needed to explain the cellular mechanism underlying the trophic and regenerative effects of BBS and NTS.

COMMENTS

Background

The exact pathogenesis in inflammatory bowel diseases is poorly understood, but some studies have analyzed the role of a variety of soluble peptides and other mediators that, once secreted in a co-ordinated fashion in the injured area, are able to restore mucosal integrity.

Research frontiers

The epithelium of the colon consists of dynamic and rapidly proliferating cells that are profoundly affected by changes in luminal contents and injury. Previous experimental studies have shown intrarectal instillation of 2,4,6- trinitrobenzene sulphonic acid (Sigma, St. Louis, Missouri) mixed with 30% ethanol (TNBS-E) promotes morphologic alterations in the colonic mucosa indicative of atrophy, increases apoptosis and induces intestinal oxidative stress. Treatment with bombesin and neurotensin attenuated colitis, as shown by the lower serum levels of tumor necrosis factor-alpha and interleukin-6, and the reduced colonic tissue levels of myeloperoxidase, malondialdehyde, carbonyls and caspases.

Innovations and breakthroughs

This study demonstrates the gut trophic peptides bombesin and neurotensin improve TNBS-E-induced ulcerative colitis. This effect related to a regulatory effect of peptides on epithelial cell production and apoptosis, and reduction of intestinal oxidative stress. Although the results of laboratory experiments are not readily applicable to the clinical situation, these findings offer further insight into the pathophysiology of gut barrier functions and suggest an important biologic effect of regulatory peptides.

Applications

The results from this experimental study confirm the protective effect of bombesin and neurotensine on TNBS-E-induced colitis and apoptosis. This encourages us to apply these agents in treating inflammatory bowel diseases in the future clinical trials.

Terminology

Bombesin and neurotensin exert a wide spectrum of biologic actions on gastrointestinal tissues, influencing intestinal motility, blood flow, secretion, absorption and immune responses. These agents are small molecules that, in the intestine, modulate several biological processes and modify the activity of cells responsible for promoting healing.

Peer review

This is a well-written manuscript. The authors describe the effect of ameliorative effects of bombesin (BBS) and neurotensin (NTS) on trinitrobenzene sulphonic acid-induced colitis.

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RAPID COMMUNICATION

Non-invasive diagnosis of *H pylori* infection: Evaluation of serological tests with and without current infection marker CIM

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Abstract

AIM: To evaluate the performance of commercially available immunochromatographic (ICT) and immunoblot tests covering the current infection marker CIM and conventional ELISA for the diagnosis of *H pylori* infection in adult dyspeptic patients.

METHODS: Consecutive non-treated dyspeptic patients undergoing diagnostic endoscopy were tested for *H pylori* infection by culture, rapid urease test, and histology of gastric biopsy specimens. Serum from 61 *H pylori* infected and 21 non-infected patients were tested for anti-*H pylori* IgG antibodies by commercial ELISA (AccuBind™ ELISA, Monobind, USA), ICT (Assure® *H pylori* Rapid Test, Genelabs Diagnostics, Singapore), and immunoblot (Helico Blot 2.1, Genelabs Diagnostics, Singapore) assays. ICT and immunoblot kits cover CIM among other parameters and their performance with and without CIM was evaluated separately.

RESULTS: Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of ELISA were 96.7%, 42.8%, 83.1%, 81.8%, and 82.9%, of ICT were 90.1%, 80.9%, 93.2%, 73.9%, and 87.8%, of ICT with CIM were 88.5%, 90.4%, 96.4%, 73.0%, and 89.0%, of immunoblot were 98.3%, 80.9%, 93.7%, 94.4%, and 93.9%, and of immunoblot with CIM were 98.3%, 90.4%, 96.7%, 95.0%, and 96.3%, respectively.

CONCLUSION: Immunoblot with CIM had the best performance. ICT with CIM was found to be more specific and accurate than the conventional ELISA and may be useful for non-invasive diagnosis of *H pylori* infection.

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Key words: *H pylori*; ELISA; Immunochromatographic test; Immunoblot; Current infection marker

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INTRODUCTION

H pylori causes peptic ulcer disease[1,2] that can be cured by antimicrobial treatment[3-5]. 'Test and treat' strategy that involves non-invasive testing without endoscopy and eradication therapy in young patients[6] is effective in management of dyspepsia[7-9]. This calls for a simple, reliable and non-invasive diagnostic test for *H pylori* infection in clinical practice. At present there is no single test for *H pylori* that can be used as the 'gold standard'[10].

Culture, rapid urease test, and histology require endoscopic biopsy of gastric mucosal tissue that is expensive, inconvenient for the patient and available only at specialized centers. Moreover, because of a patchy distribution of *H pylori* in the gastric mucosa, biopsy tissue examination may yield false negative results[11]. Serological tests that detect anti-*H pylori* IgG antibodies are non-invasive, less expensive, not influenced by sampling error, and less likely to be confounded by suppression of *H pylori* infection by colloidal bismuth, proton pump inhibitors, or antibiotics[12]. Serological tests are widely used[13] but they cannot differentiate a current infection from a past exposure[10]. Performance of serological tests depends on the antigen preparation used[12,14], and as *H pylori* strains differ among geographic locations, local validation of the test is necessary[13,14]. A recent development in *H pylori* diagnosis is a commercial immunochromatographic test (ICT) and an immunoblot test covering the current infection marker CIM. CIM is an antigenic protein synthesized by recombinant DNA technology. It is homologous to a conserved secreted protein of *H pylori*. According to its manufacturer (Genelabs Diagnostics, Singapore), presence of anti-CIM IgG antibody is highly predictive for active *H pylori* infection. If so, it should be helpful for diagnosis of *H pylori* infection where facility for endoscopy is not available. Several studies have found the immunoblot test Helico Blot 2.0, its newer version Helico Blot 2.1, and ICT kit Assure® *H pylori* Rapid Test, all of which are able to detect anti-CIM antibodies, effective for diagnosis of *H pylori* infection in adults and children[15-20]. Bangladesh is a developing country with a high prevalence of *H pylori* infection and peptic ulcer[21,22]. Different tests have been evaluated for diagnosis of *H pylori* infection in Bangladesh[23-26]. Two studies involved in-house immunoblot assays[25,26]. To the best of our knowledge standardized commercial immunoblot or ICT tests with CIM have not been evaluated in Bangladesh. The aim of this study was to evaluate the performance of three commercially available serological tests based on three methods to find a reliable serological test for non-invasive diagnosis of *H pylori* infection in adult dyspeptic patients: ELISA (AccuBind™, ELISA, Monobind, USA), ICT (Assure®, *H pylori* Rapid Test, Genelabs Diagnostics, Singapore), and immunoblot (Helico Blot 2.1, Genelabs Diagnostics, Singapore). ICT and immunoblot assays cover CIM. The performance of ICT and immunoblot tests was evaluated separately with and without CIM to see whether CIM improves performance of these tests.

MATERIALS AND METHODS

Patient selection

Consecutive adult dyspeptic patients attending the Department of Gastrointestinal, Hepatobiliary and Pancreatic Diseases (GHPD) of Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) for diagnostic endoscopy during June 2004 to January 2005 were selected. Informed consent was obtained from each patient before endoscopy, and sample collection and approval of the Ethical Review Committee of BIRDEM was taken prior to initiation of the project work. Patients who underwent partial or complete gastrectomy, or those with a prior *H pylori* eradication therapy, or those who were treated with any antibiotics, colloidal bismuth compounds, proton pump inhibitors, or H2 receptor blocker within the last four weeks were excluded.

Endoscopy and gastric biopsy

Endoscopy was done with an Olympus EVIS 160 video endoscope (Olympus Optical Company, Japan) after an overnight fast. Six gastric biopsies (three from antrum and three from corpus) were taken from each patient.

Collection of serum

After endoscopy 3 mL of venous blood was collected from each patient. Serum was separated after 1 h and kept at -70℃ until serological tests were performed.

Culture

Two gastric biopsy specimens, one from the antrum and one from the corpus, were taken up into Stuart's transport media. Cooled samples were transported to the *H pylori* Laboratory of Laboratory Sciences Division of International Centre for Diarrhoeal Diseases Research, Bangladesh (ICDDR,B) within 3-4 h where culture was done as described previously[27]. Positive cultures were identified by colony and Gram stain morphology and positive catalase, oxidase, and urease tests.

Rapid urease test (RUT)

Two gastric biopsy specimens, one from the antrum and one from the corpus, were taken up in Christensen's urea agar media (pH adjusted at 7.0) in screw-capped bottles. A change of color from yellow to pink by any specimen within 2 h was considered as positive.

Histology

Two gastric biopsy specimens, one from the antrum and one from the corpus, were fixed in 10% formalin in separate containers and were sent to the Histopathology Laboratory of Ibrahim Medical College. Samples were embedded in paraffin wax, cut at 5 μm thickness and were stained by modified Giemsa and hematoxylin & eosin (HE) dye. *H pylori* was identified from its characteristic appearance and distribution. The histopathologist (Nasim Ahmed) was unaware of patients' clinical conditions and other test results.

'Gold standard' definition of H pylori infection

As there is no single 'gold standard' test for *H pylori*, an operational 'gold standard' definition of *H pylori* infection was used. The definition was as follows: Patients with positive culture result were considered as infected. In the case of a negative culture, patients positive by both RUT and histology were considered as infected. Patients negative by all three gastric biopsy specimen based tests were considered as non-infected. Patients negative by culture and positive by either RUT or histology were considered as indeterminate[16,17].

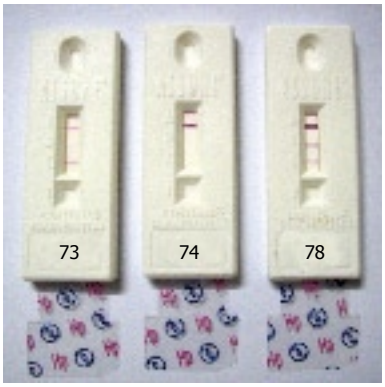


Figure 1 Photograph of serum ICT by Assure® *H pylori* Rapid Test. Left: ICT positive; Middle: Negative; Right: ICT with CIM positive (73, 74, and 78: Patients' identification numbers).

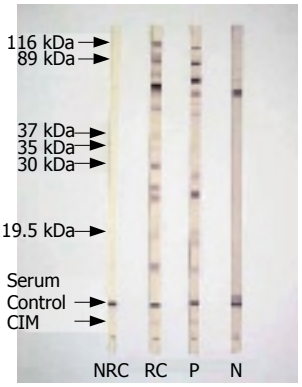


Figure 2 Photograph of serum Immunoblot test by Helico Blot 2.1. kDa: kilo Dalton; CIM: Current infection marker; NRC: Non-reactive control; RC: Reactive control; P: Positive Immunoblot with CIM; N: Negative.

ELISA

ELISA test for anti-*H pylori* IgG antibody was performed using a commercial test kit, AccuBind™ ELISA (Monobind, USA), according to instructions of the manufacturer.

ICT

ICT test was performed by the commercial test kit Assure® *H pylori* Rapid Test (Genelabs Diagnostics, Singapore) according to the instructions of the manufacturer. When control (A) and test line (C) were visible the ICT test was regarded positive, when control (A), test (C), and CIM line (B) were visible it was regarded ICT with CIM positive. When only control line (A) was visible, the test was regarded negative (Figure 1). When control line (A) was absent or control line (A) and CIM line (B) present but test line (C) absent the test was regarded to be invalid.

Immunoblot

Immunoblot test was done using the commercial kit Helico Blot 2.1 (Genelabs Diagnostics, Singapore). Helico Blot 2.1 consists of a Western Blot made from bacterial lysate of *H pylori* strain ATCC 49503 and a recombinant antigen called CIM[18]. The test strip contains *H pylori* antigens with molecular weights of 116 kDa (CagA), 89 kDa (VacA), 37 kDa, 35 kDa, 30 kDa (Urease A), and 19.5 kDa as separate lines. CIM is located at the bottom of the strip as an independent band. The test was done and interpreted according to instructions of the manufacturer (Figure 2). The manufacturer's recommended criteria for determining *H pylori* positivity by Helico Blot 2.1 was as follows: (1) 116 kDa (CagA) positive, where CagA has to be present with at least one of the following bands - 89 kDa (VacA), 37 kDa, 35 kDa, 30 kDa (UreA), or 19.5 kDa, or with CIM, (2) presence of any one band at 89 kDa, 37 kDa, or 35 kDa, with or without CIM, (3) presence of both 30 kDa and 19.5 kDa band with or without CIM.

ELISA, ICT, and immunoblot tests were carried out at the Immunology Laboratory of the Department of Immunology of BIRDEM.

Calculation of performance

Samples from 62 patients who were infected and 21 patients who were non-infected according to 'gold standard' definition were subjected to serological testing. Eight patients were indeterminate and excluded. Results of serological tests were plotted against *H pylori* infection status, and sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy with 95% confidence interval were calculated using standard formula.

RESULTS

Patients' characteristics

A total of 82 patients were included in the study. They were aged between 18 to 75 years with a mean age of 47.6 years and standard deviation 14.0 years. Patients' characteristics are shown in Table 1.

H pylori positivity by different tests

Of the 82 patients, 53 (64.6%) were culture positive, 40 (48.8%) were rapid urease test positive, 31 (37.8%) were histology positive, 71 (86.5%) were ELISA positive, 59 (71.9%) were ICT positive, 56 (68.2%) were ICT with CIM positive, 64 (78.0%) were immunoblot positive, and 62 (75.6%) were immunoblot with CIM positive.

Performance of serological tests

Sensitivity, specificity, PPV, NPV, and accuracy of serological tests were calculated from data presented in Table 2, and the calculated performance is shown in Table 3. ELISA was very sensitive but its specificity was low. Sensitivity and NPV of ICT were lower but specificity, PPV, and accuracy were much higher than ELISA. CIM decreased sensitivity of ICT slightly but increased its specificity, PPV, and accuracy. Sensitivity of immunoblot was higher than ELISA or ICT. Its specificity was higher than ELISA and same as ICT. Its PPV, NPV, and accuracy were much higher than ELISA and ICT. CIM increased specificity, PPV, NPV, and accuracy of immunoblot without altering its sensitivity (Table 3).

DISCUSSION

Since its discovery many tests have been designed for diagnosis of *H pylori*[12]. But no test is accurate enough to be the 'gold standard'[10]. Serological tests are widely used for non-invasive diagnosis[13] but a positive serological test does not mean active infection[10]. To find a reliable non-invasive test for *H pylori*, we evaluated commercially available conventional ELISA, and new ICT and immunoblot assays covering the recombinant CIM.

Table 1 Characteristics of selected patients (n = 82)

Characteristics	n	Percentage
Age group (yr)		
≤ 30	11	13.4
31-40	15	18.3
41-50	21	25.6
51-60	22	26.8
≥ 60	13	15.9
Sex		
Male	49	59.8
Female	33	40.2
Smoking habit		
Smoker	25	30.5
Non-smoker	57	69.5
Alcohol intake		
Take alcohol	3	3.7
Do not take alcohol	79	96.3
Diabetes		
Diabetic	67	81.7
Non-diabetic	15	18.3
Endoscopic diagnoses		
Normal	20	24.4
Gastritis	24	29.3
Duodenitis	8	9.8
Peptic ulcer	25	30.5
Gastric carcinoma	4	4.8
Reflux esophagitis	1	1.2

Table 2 Comparison of serological tests with *H pylori* infection (n = 82)

Test	*H pylori* infection[1]	
	Infected (n = 61) n (%)[2]	Non-infected (n = 21) n (%)[2]
ELISA		
Positive (n = 71)	59 (83.0)	12 (17.0)
Negative (n = 11)	2 (18.2)	9 (81.8)
ICT		
Positive (n = 59)	55 (93.2)	4 (6.8)
Negative (n = 23)	6 (26.1)	17 (73.9)
ICT with CIM		
Positive (n = 56)	54 (96.4)	2 (3.6)
Negative (n = 26)	7 (26.9)	19 (73.1)
Immunoblot		
Positive (n = 64)	60 (93.8)	4 (6.2)
Negative (n = 18)	1 (5.6)	17 (94.4)
Immunoblot with CIM		
Positive (n = 62)	60 (96.8)	2 (3.2)
Negative (n = 20)	1 (5.0)	19 (95.0)

[1]Infected: Culture positive or culture negative but both RUT and histology positive; non-infected: All three tests negative; [2]Percentages are over row total.

The commercial ELISA that we evaluated was very sensitive but less specific (Table 3). Performance of the ELISA kit varies in different populations. Laheij *et al* reviewed a range of sensitivity of 57%-100% and a range of specificity of 31%-100% for different commercial kits in different populations[28]. A study in the Netherlands evaluated eight commercial ELISA tests and found sensitivities of 93%-98% and specificities of 95%-98%[29]. Using a further commercial kit, a study from Bangladesh found sensitivity, specificity, PPV, and NPV of 100%, 13.6%, 54.8%, and 100%, respectively[24].

Performance of ICT with CIM evaluated in this study was better than two other commercial ICT kits evaluated in Chinese patients[30] and similar to another commercial ICT kit evaluated in African-American, Caucasian, and Asian population with ^{13}C urea breath test as the 'gold standard'[31]. Its performance in our study was lower than a study that evaluated this kit in Thai children[19] and similar to a study that evaluated this kit in Portuguese children[20].

Performance of immunoblot with CIM in our study was similar to reports that evaluated the same kit in adult French population[16] and Portuguese children of different age groups[17]. Its specificity was lower in our study than a study in Japanese and American population[18]. Lepper *et al* compared different immunoblot kits and found sensitivity, specificity, PPV, and NPV 62.8%-95.9%, 85.7%-100.0%, 97.2%-100.0%, and 37.7%-82.4%, respectively[32].

Among the serological tests evaluated, immunoblot with CIM had the highest sensitivity, specificity, PPV, NPV, and accuracy (Table 3). It thus may be useful for non-invasive diagnosis of *H pylori* infection. Though conventional ELISA was highly sensitive it may not be reliable for diagnostic purpose in these patients due to its low specificity. Immunoblot is costly, takes longer time, and requires laboratory set-up and a trained staff. Though ELISA is cheaper than immunoblot, it also requires laboratory set-up and a trained staff. ICT with CIM had sensitivity and NPV lower than immunoblot or ELISA, but its specificity, PPV, and accuracy were higher than ELISA. Considering sensitivity, specificity, PPV, and accuracy, ICT with CIM yielded good performance (Table 3). ICT is a rapid test, easy to carry out, and can be done at the physician's chamber or in the field level where well-equipped laboratory and trained staff is not available.

In conclusion, immunoblot with CIM had the best performance. Though the sensitivity of ICT with CIM was slightly lower, due to its high specificity and accuracy it would be a more reliable diagnostic tool than conventional ELISA. As ICT with CIM has good performance and it is cheap, easy and rapid, it may be useful for non-invasive diagnosis of *H pylori* infection in adult dyspeptic patients of Bangladesh.

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COMMENTS

Background

A reliable non-invasive test for *H pylori* is essential in clinical practice. Commercial serological tests based on enzyme linked immunosorbent assay (ELISA), immunochromatographic test (ICT) and immunoblot are widely used. But they are unable to distinguish between active infection and a previous contact. Gastric biopsy based tests can indicate active infection but they are inconvenient for the patients and not available everywhere. A recent development is commercial ICT and immunoblot tests with CIM that may indicate active *H pylori* infection. In this study we evaluated performance of commercial ELISA without CIM and ICT and immunoblot with CIM for pre-treatment diagnosis of *H pylori* infection.

Research frontiers

CIM is an antigenic protein synthesized by recombinant DNA technology.

Table 3 Performance of serological tests

Test	Performance (95% Confidence interval) in percentage				
	Sensitivity	Specificity	PPV[1]	NPV[2]	Accuracy
ELISA	96.7 (88.6-99.4)	42.8 (21.8-65.9)	83.1 (72.3-90.9)	81.8 (48.2-97.7)	82.9 (73.0-90.3)
ICT	90.1 (79.8-96.3)	80.9 (58.0-94.5)	93.2 (83.5-98.1)	73.9 (51.5-89.7)	87.8 (78.7-93.9)
ICT with CIM	88.5 (77.7-95.2)	90.4 (69.6-98.8)	96.4 (87.6-99.5)	73.0 (52.2-88.4)	89.0 (80.1-94.8)
Immunoblot	98.3 (91.2-99.9)	80.9 (58.0-94.5)	93.7 (84.7-98.2)	94.4 (72.7-99.8)	93.9 (86.3-97.9)
Immunoblot with CIM	98.3 (91.2-99.9)	90.4 (69.6-98.8)	96.7 (88.8-99.6)	95.0 (75.1-99.8)	96.3 (89.6-99.2)

[1]PPV: Positive predictive value; [2]NPV: Negative predictive value.

According to its manufacturer, presence of anti-CIM IgG antibodies indicates active *H pylori* infection. It is interesting because usually active infection cannot be diagnosed from serum IgG response to an organism.

Innovations and breakthroughs

Though the commercial kits used in this study may not represent the methods, from this study we can see both ICT and immunoblot with CIM have better performance than conventional ELISA evaluated, and CIM improves performance of both ICT and immunoblot kits. We can also see that performance of CIM varies depending on the method in which it is used. It performed better in immunoblot than in ICT.

Applications

This study will facilitate diagnosis of *H pylori* infection.

Peer review

In this clinical report, Dr. Rahman *et al* evaluated the performance of serological tests with current infection marker and ELISA for the non-invasive diagnosis of *H pylori* infection. Informative results were obtained from their study which could benefit other physicians. Therefore, this study is of importance.

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RAPID COMMUNICATION

Hepatitis C virus genotypes distribution and transmission risk factors in Luxembourg from 1991 to 2006

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Abstract

AIM: To analyze the Hepatitis C virus (HCV) genotype distribution and transmission risk factors in a population of unselected patients in Luxembourg.

METHODS: Epidemiological information (gender, age and transmission risks) were collected from 802 patients newly diagnosed for hepatitis C and living in Luxembourg, among whom 228 patients referred from prison. Genotyping using 5'noncoding (5'NC) sequencing was performed. We compared categorical data using the Fisher's exact *F*-test and odds ratios (OR) were calculated for evaluating association of HCV genotype and risk factors.

RESULTS: The sex ratio was predominantly male (2.2) and individuals aged less than 40 years represented 49.6% of the population. Genotype 1 was predominant (53.4%) followed by genotype 3 (33%). Among risk factors, intravenous drug usage (IVDU) was the most frequently reported (71.4%) followed by medical-related transmission (17.6%) including haemophilia, transfusion recipients and other nosocomial reasons. Genotype 3 was significantly associated to IVDU (OR = 4.84, $P < 0.0001$) whereas genotype 1 was significantly associated with a medical procedure (OR = 2.42, $P < 0.001$). The HCV genotype distribution from inmate patients differed significantly from the rest of the population (Chi-square test with four degrees of freedom, $P < 0.0001$) with a higher frequency of genotype 3 (46.5% *vs* 27.5%) and a lower frequency of genotype 1 and 4 (44.7% *vs* 56.8% and 5.3% *vs* 9.6%, respectively). IVDU was nearly exclusively reported as a risk factor in prison.

CONCLUSION: We report the first description of the HCV genotype distribution in Luxembourg. The repartition is similar to other European countries, with one of the highest European prevalence rates of genotype 3 (33%). Since serology screening became available in 1991, IVDU remains the most common way of HCV transmission in Luxembourg.

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Key words: Hepatitis C virus; Genotypes; Luxembourg; Risk factors; Substance abuse; Prisons

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INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver disease and hepatocellular carcinoma worldwide. The most recent WHO estimate of the prevalence of HCV infection is 2%, ranging from 0.6% to 2.3% in North America, Northern and Western Europe and Australia[1]. HCV has a high molecular diversity worldwide. A recent international standardization of the nomenclature proposed a classification into 6 genotypes (1 to 6) and more than 70 subtypes (termed a, b, c, d,…)[2].

Although genotypes 1, 2 and 3 are responsible for more than 90% of the infections in North and South America, Europe and Japan[3], prevalence and distribution peculiarities of HCV genotypes are linked to geographical location and mode of transmission[4]. Three broad patterns

of genotype distribution have been identified to date[4]. One pattern, characterized by high genetic diversity, involves geographically discrete areas where HCV has been endemic for a long time such as West Africa with types 1 and 2, Central Africa with type 4, and Asia with types 3 and 6. Another pattern involves areas with a few subtypes circulating in specific risk groups, e.g. subtype 3a in drug addicts. The third pattern involves areas where a single subtype is present, such as in Egypt with subtype 4a and South Africa with subtype 5a.

Beside epidemiological considerations, HCV genotyping also has a clinical impact. Treatment for chronic hepatitis C includes standard or pegylated (PEG) interferon-α (IFN-α) in combination with ribavirin[5,6]. HCV genotype is a strong independent predictor of sustained virological response, which is defined by an undetectable HCV RNA level 24 wk after the end of treatment[7]. Genotypes 2 and 3 are significantly associated with a better therapeutic response, and knowledge on the HCV genotype is now used for tailoring therapeutic modalities: the duration of treatment is longer (48 wk) for genotype 1, 4, 5 and 6 than for genotype 2 and 3 (24 wk).

Up to now, no population-based study has been undertaken in Luxembourg and the precise prevalence rate of HCV infected people is not known. The National Council of Communicable Diseases in 2006 estimated the prevalence of HCV infection to be between 0.5% and 1%. Therefore, between 2250 and 4500 individuals might be infected in Luxembourg. Identifying risk factors is important to plan preventive activities and is necessary to target the higher risk population. HCV spreads in humans mainly through parenteral transmission. While uncontrolled medical procedures or transfusion were major causes of HCV transmission before serology screening was available, intravenous drug use (IVDU) has now become the predominant source of infections in developed countries[8]. Transmission of HCV also occurs through occupational, perinatal or sexual exposures, albeit with much less efficiency. Practices such as non-injection drug use (particularly via sharing of non-injecting drug equipment), tattooing or body piercing have also emerged as additional causes of HCV transmission[9].

The relation between incarceration and the high transmission of blood-borne viruses has been known for several years[10-12]. The HCV prevalence rate in prison is higher than in the general population because of the concentration of injecting drug users. HCV screening and genotyping was introduced in prisons of Luxembourg in 2002. These patients are usually referred to the Centre Hospitalier of Luxembourg (CHL) for medical treatment.

Because of its impact on patient management and treatment, we made a retrospective analysis of the HCV genotype distribution in a population of 802 unselected patients living in Luxembourg using 5'noncoding (5' NC) sequencing. We also analysed transmission risks related to infection. As almost 30% of the patients live in prison, we distinguished this population in our analysis. Our survey gives the first estimation of the distribution of HCV genotypes amongst unselected HCV patients in Luxembourg.

MATERIALS AND METHODS

Patients

Genotype and epidemiological information (gender and age) was collected from 802 patients newly diagnosed for hepatitis C and referred to the Centre Hospitalier of Luxembourg from 1991 to 2006. Among the overall population of 802 patients, a subset of 228 (28.4%) referred from prison (Centre Pénitentiaire of Luxembourg or CPL) has been distinguished from the remaining 574 patients, the latter being considered as general population. Among the 802 patients, 60 (7.6%) were also chronically co-infected with HIV-1 and 7 (0.9%) with HBV. Information regarding risk factors [intravenous drug usage (IVDU), medical reason (including transfusion and haemophilia or other nosocomial reasons), sexual transmission, mother-to-child transmission, occupational exposure, tattooing and body piercing] was available for 437 of the 802 patients (54.5%), and for 103 of the 228 patients (45.2%) of the CPL.

Methods

Detection of sera specific antibodies to HCV (ABBOTT Axsym HCV v3.0, Abbott, Louvain-la-Neuve, Belgium), antigen/antibodies to HIV (HIV Combo v4.0, Abbott) and surface HBV antigens (Ag HBs v2.0, Abbott) was performed with commercial kits. HCV viral load was determined with the quantitative COBAS AMPLICOR® HCV test v2.0 (Roche Diagnostics, Mannheim, Germany). Only samples with a viral load superior to 20 000 IU/mL were considered for genotyping. PCR products generated with the COBAS AMPLICOR® HCV test were collected for subsequent genotyping using the TRUGENE® HCV 5' NC methodology (Bayer s.a., Brussels, Belgium).

HCV genotype incidence

The incidence of HCV genotypes in our cohort has been calculated, per year, as the proportion of the number of new included genotypes divided by the total number of new included genotypes. Results are expressed as percentages. Patient inclusion in our cohort is based on the date of the first viral load analysis. Determination of the incidence has been performed from 1999 to 2006.

Statistical analysis

The Fisher's exact test was used to compare categorical data such as genotype, age and gender. Odds ratios were calculated for evaluating association of HCV genotype with risk factor. Stratified analyses were conducted using the Mantel-Haenszel estimator for adjusting odds ratios with age and gender.

RESULTS

Gender and age

The sex ratio (SR) of the 802 investigated patients was predominantly male (2.2) (Table 1). Individuals aged less than 40 years, for whom a more recent time point of infection could be assumed, represented 49.6% of all patients. The population of HCV-infected patients at CPL was almost exclusively male (94.7%, SR = 17.8), with a

significantly larger proportion of younger patients (77.6% patients aged less than 40 years, compared to 38.5% in the rest of the population, $P < 0.0001$).

Genotype distribution

Genotype 1 was predominant, with 428 patients (53.4%), followed by genotype 3 (33%), 4 (8.3%), 2 (4.6%) and 5 (0.6%) (Table 1). Among patients co-infected with HIV or HBV, the genotype distribution was similar to the overall population (data not shown). Regarding prison inmates (Table 1), genotypes 1 and 3 were predominant (44.7% and 46.5%, respectively), followed by genotype 4 (5.3%) and 2 (3.5%). The HCV genotype distribution in patients from CPL differed significantly (Chi-square test with four degrees of freedom, $P < 0.0001$), with a higher frequency of genotype 3 (46.5% *vs* 27.7%) and a lower frequency of genotype 1 and 4 (44.7% *vs* 56.8% and 5.7% *vs* 9.58%, respectively).

As shown in Figure 1, the analysis of HCV genotype incidence has been performed from 1999 to 2006. The rate of genotypes 1, 2, 3 and 4 was relatively constant over time, only isolated cases of genotype 5 were observed during this 7 years period.

Distribution of genotypes according to gender and age

As described in Figure 2A, genotype 3 was significantly more frequent in males (69.1% *vs* 30.9%, $P < 0.0001$), while genotypes 2 and 5 were more frequent in females ($P = 0.047$ and $P = 0.0027$, respectively). The frequency of genotypes 1 and 4 were independent of gender and age (Figure 2A and B).

Genotype 3 was associated significantly with patients aged less than 40 years (60% *vs* 44.5% for patients aged more than 40 years, $P < 0.0001$) (Figure 2B). Conversely, Genotype 2 was significantly associated with patients aged more than 40 years (73% *vs* 49.3%, $P = 0.0064$).

Transmission risk factors

Among risk factors (Table 2), IVDU was the most frequently reported (71.4%), followed by medical-related transmission such as transfusion, haemophilia or other nosocomial reasons (17.6%, 77/437, of whom 68 cases through transfusion). 35 patients (4.7%) reported infection through sexual contact (homosexual, bisexual and heterosexual transmission). Other routes of transmission were also reported such as non-injection drug use (5 cases), occupational exposure (3 cases), tattooing (3 cases), mother-to-child transmission (1 case) and body piercing (1 case). IVDU was nearly exclusively reported as a risk factor for CPL (94.2% of the reported cases) (Table 2). Four inmate patients reported infection through medical procedure, one after sniffing and another one after tattooing. In the general population, the predominant source of infection remained IVDU (64.3%).

Association of the genotypes and transmission risk factors

Odds ratios were determined for evaluating association of HCV genotype with risk factor in the overall population (Table 3). The odds ratio for association of IVDU and HCV genotype 3 was 4.84 (95% confidence intervals, 2.86 to 8.25; $P < 0.0001$). When adjusted for age and gender, IVDU patients were still significantly more likely than other patients to be infected with HCV genotype 3 (Mantel-Haenszel adjusted odds ratio = 4.54, 95% confidence intervals, 1.60 to 12.7; $P = 0.0043$). Conversely, IVDUs were at lower risk of being infected with HCV genotype 1 whether or not they were adjusted for age and gender.

The odds ratio for association of medical procedure and HCV genotype 1 was 2.42 (95% confidence intervals, 1.43 to 4.05; $P = 0.0008$). When adjusted for age and gender, patients reporting infection through a medical procedure were still 2.34 as likely as other patients for contracting HCV genotype 1 (Mantel-Haenszel adjusted odds ratio = 2.34, 95% confidence intervals, 1.35 to 4.06; $P = 0.0025$).

Finally, when adjusted for population type, IVDU patients were still significantly more likely than other patients to be infected with HCV genotype 3 (Table 3).

Table 1 Patient details and HCV genotype distribution in the overall population, in the Centre Pénitentiaire of Luxembourg (CPL) and in the general population

Variable	Overall	CPL	General
n (%)	802 (100)	228 (28.4)	574 (71.6)
Sex ratio	2.2	17.8	1.44
Age (% less than 40 yr)	49.6	77.6	38.5
Genotype			
1	428 (53.4)	102 (44.7)	326 (56.8)
2	38 (4.7)	8 (3.5)	30 (5.2)
3	264 (33)	106 (46.5)	158 (27.5)
4	67 (8.3)	12 (5.3)	55 (9.6)
5	5 (0.6)	0	5 (0.9)

DISCUSSION

We report here the first description of HCV molecular epidemiology in Luxembourg. This survey has been realized with a relatively confident case number of 802 unselected patients seen in routine practice, irrespective of their clinical background. The genotype distribution is similar to other European countries with yet one of the highest European prevalence of genotype 3 (33%). Injecting drug use has currently become the principal cause of HCV transmission.

HCV genotypes 1 and 3 were the most prevalent in our patients. In Europe, there have been dynamic changes over time in the prevalence of the different HCV subtypes. The prevalence of HCV 1b and 2 have decreased, while HCV 1a , 3a and 4 have increased[13-15]. Regarding incidence of genotypes in Luxembourg, we did not notice significant changes in the emergence of genotype 3 from 1999 to 2006. The genotype proportion, as observed in our cohort, has been relatively constant overtime. No new genotype has been massively introduced in Luxembourg despite strong progression of the immigration flow from 1985 to 2001. One reason might be that the majority of immigrants are coming from the European Union. Because of the few data available, we were not able to find

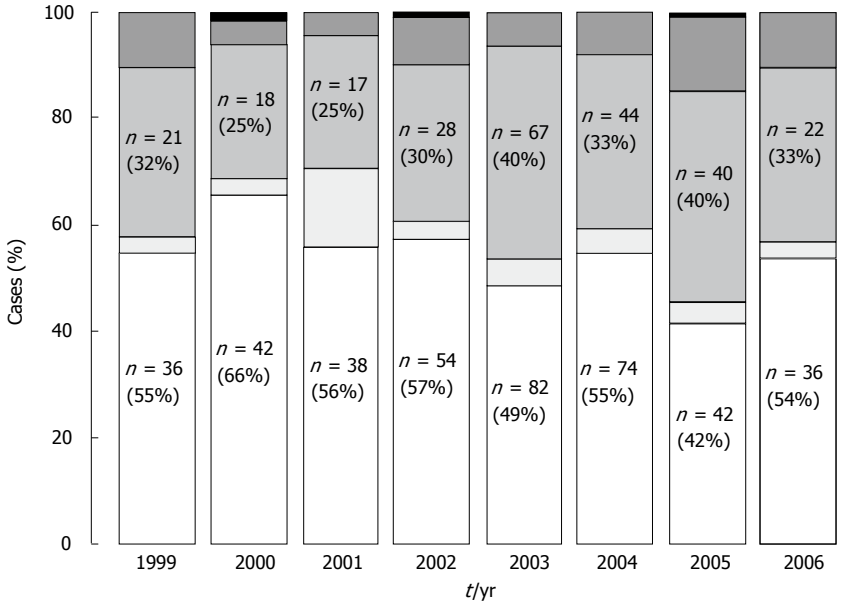


Figure 1 HCV genotype incidence from 1999 to 2006. The genotypes are indicated in the bars by different shades of grey, representing from bottom to top, genotypes 1, 2, 3, 4 and 5. Number of cases and percentages are mentioned for genotype 1 and 3. No significant changes in genotype distribution over time have been observed during this 7 years period.

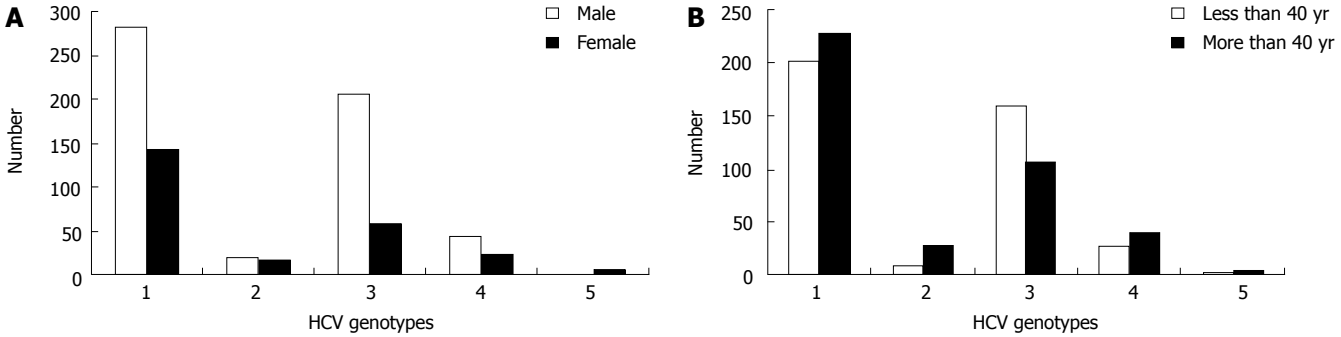


Figure 2 Distribution of HCV genotypes. **A**: Distribution of HCV genotypes in patients according to gender. Genotype 3 is more frequent in male ($P < 0.0001$), genotype 2 and genotype 5 are more frequent in female ($P = 0.047$ and $P = 0.0027$ respectively); **B**: Distribution of HCV genotypes in patients according to age. Genotype 3 is associated to patients < 40 yr ($P < 0.0001$). Genotype 2 is associated to patients > 40 yr ($P = 0.0064$).

any association of genotypes and demographic data for the patients. As for the several genotype 4 viruses identified in our cohort, we can assume that their introduction could be related to African immigration[16]. One limitation of our study is the methodology used for genotyping: the TRUGENE® HCV 5'NC method indeed does not allow for easy discrimination between HCV subtypes. Therefore, the apparent invariability of HCV molecular epidemiology may mask significant variability in terms of subtypes.

The high prevalence of HCV genotype 3 in our population is attributed to IVDU in the overall population and in particular in the incarcerated population. In similar studies from neighbouring countries, distribution rate of genotype 3 varied from 13.42% in Germany[14], 22%-26.3% in France[13,17] to 29.3% in the Netherlands[18]. In prison, injecting drug use is the most commonly reported risk factor[19-21]. In the rest of the population, the prevalence of genotype 3 is 27.5%. This rate is comparable with other studies[13,14,17,18] in which inclusion of prisoners was not specified. Other molecular epidemiology studies have shown that HCV subtype 3a is significantly associated with transmission through injecting drug use in industrialized countries[22-24] and explain the prevalence of subtype 3a in many North and South American and European countries. HCV subtype 3a originates from Asia and has spread widely among injecting drug users[25,26]. Mechanisms of subtype 3a transmission remain poorly defined. Genotype 3 was not associated with a high level of HCV RNA as compared to the others genotypes in our cohort. Nevertheless, the relative homogeneity of NS5B sequences of injecting drug users from France, South America, Australia and California have indicated that HCV subtype 3a has been transmitted from a common origin through a unique worldwide epidemic among the drug user communities[27]. In this study, the authors reported region-specific HCV 3a variants suggesting local transmission among intravenous drug users from the same geographical area. Therefore, the intrinsic characteristics of the transmission network of IVDU (unsafe injecting practices, drug preparation materials, needle or syringe exchange) together with the importance of the social context of drug injections may explain the efficiency of HCV genotype 3 transmission in Luxembourg.

Our study shows that the main risk factor for infection by genotype 3 is IVDU whereas the main risk factor for infection by genotype 1 is medical-related transmission.

Table 2 Transmission risk factors in the overall population, in the Centre Pénitentiaire of Luxembourg (CPL) and in the general population

Risk factor	Overall	CPL	General
n (%)	437 (100)	104 (23.8)	333 (76.2)
IVDU	312 (71.4)	98 (94.2)	214 (64.3)
Medical-related	77 (17.6)	4 (3.8)	73 (21.9)
Sexual	35 (8)	0	35 (10.5)
Other	13 (3)	2 (2)	11 (3.3)

Table 3 Associations between genotype and transmission risk factors for the overall population

Risk factor	IVDU	Medical-related
Odds ratios (OR)		
Genotype 1	0.50 (0.33 to 0.77, $P = 0.0016$)	2.42 (1.43 to 4.05, $P = 0.0008$)
Genotype 3	4.84 (2.86 to 8.25, $P < 0.0001$)	0.17 (0.09 to 0.35, $P < 0.0001$)
MH adjusted OR[1]		
Genotype 1	0.49 (0.30 to 0.84, $P = 0.0084$)	2.34 (1.35 to 4.06, $P = 0.0025$)
Genotype 3	4.54 (1.60 to 12.70, $P = 0.0043$)	0.21 (0.07 to 0.64, $P = 0.0064$)
MH adjusted OR[2]		
Genotype 1	0.55 (0.35 to 0.85, $P = 0.0081$)	2.24 (1.31 to 3.84, $P = 0.0033$)
Genotype 3	4.28 (2.42 to 7.57, $P < 0.0001$)	0.20 (0.09 to 0.42, $P < 0.0001$)

Odds ratio with confidence intervals 95%. [1]Mantel-Haenszel odds ratio adjusted for age and gender; [2]Mantel-Haenszel odds ratio adjusted for population type.

These data are in agreement with other studies in Europe[15,28-30]. Serology screening is routinely performed since the early 1990s and recent infections are more likely attributable to other risk factors rather than medical-related transmission (blood, blood transfusion or surgery). Interestingly, 4 patients from the general population reported infections through tattooing and body piercing. This finding reemphasizes the fact that practices using sharps in personal services are potential sources of spread of infection.

The age of infected individuals may be a direct variable for the genotype distribution. Nevertheless, our data did not show that patients infected by genotype 1 were older than 40 years. This would mean other routes of infection by genotype 1 such as IVDU. Since serology screening is available, IVDU remains the more common route of HCV transmission in Luxembourg. Genotype 3 is significantly associated with males aged less than 40 years which is consistent with the profile of drug injecting users. The male/female ratio of the IVDU population is 3:1 and the mean age of indexed intravenous drug users is about 30 years in Luxembourg. We found that genotype 2 is significantly associated with patients aged more than 40 years. These results may indicate that the type 2 infections were not recent but probably linked to some distant event like blood transfusion. Unfortunately, we could not find any correlation of this genotype with a route of transmission. In addition, we have also shown that genotype 2 and 5 are more frequent in female. To our knowledge, this association has never been reported and merits further investigation.

Incarcerated individuals have high rates of infectious diseases. The concentration of a marginal population in prison has necessitated systematic and rigorous screening for hepatitis B and hepatitis C. Consistent with similar studies worldwide, the prevalence of HCV in prison inmates in CPL (23.7% in 2006) is higher than in the general population suggesting probable transmission in prisons through intravenous drug use and unsafe sexual behaviour[12,21,31,32]. In CPL, the prevalence rate of HCV has increased from 19.7 % in 2001 to 23.7% in 2006. This increase is consistent with the continuous increase of infection rate in the IVDU population observed in Luxembourg as well as other European countries[33]. HCV among injecting drug users is one of the European Union's major public health problems. In 2004, statistics from our laboratory provided the proportion of 74% reported HCV infections with acute and chronic HCV infections in the IVDU population. Therefore, prisons provide a good location to offer HCV screening and treatment and to prevent future transmission. Sharing injection equipment as well as tattooing and piercing during incarceration among intravenous drug users are more frequent than in the community[34]. In this respect, a syringe/needle exchange program was implemented in CPL in 2005. Exchange is conducted through the medical unit and requires an initial evaluation by the prison doctors.

In conclusion, our study gives the first estimation of the genotype distribution in Luxembourg. Like in other countries from the European Union, IVDU is the driving force for HCV transmission in Luxembourg. Therefore, increased prevention efforts targeted to drug injection are urgently needed to curb the epidemic. HCV genotype distribution has to be followed in the future in order to improve the biological tests for diagnosis and the development of preventive procedures among IVDU or the prison population. As the genotype distribution has a direct impact on medical practice and treatment, it may require new therapeutic strategies in particular with regards to vaccine development.

COMMENTS

Background

Hepatitis C virus (HCV) has a high molecular diversity worldwide and is classified into 6 genotypes. Prevalence and distribution peculiarities of HCV genotypes are linked to geographical location and mode of transmission.

Research frontiers

Data on genotypic distribution from various areas are crucial to understand the spreading mode of HCV. Identifying risk factors is important to plan preventive activities and is necessary to target the higher risk population.

Innovations and breakthroughs

This report describes for the first time the HCV genotype distribution in Luxembourg. The repartition is similar to other European countries, with one of the highest European prevalence rates of genotype 3 (33%) which is associated to intravenous drug usage.

Applications

HCV among injecting drug users is one of the European Union's major public health problems. Mechanisms of genotype 3 transmission remain poorly defined and need to be investigated to explain the efficiency of HCV genotype 3 transmission in Luxembourg.

Peer review

This article is interesting. The authors have provided valuable information about epidemiological aspects of HCV genotype distribution in Luxembourg with nice connections to the similar studies worldwide.

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RAPID COMMUNICATION

H pylori: Treatment for the patient only or the whole family?

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Abstract

AIM: To compare the effects of treatment of *H pylori*-infected individuals with the effects of treatment of individuals as well as all *H pylori*-infected family members.

METHODS: *H pylori*-positive patients with similar demographic specifications were prospectively randomized with respect to treatment, with a triple regimen of either patients and all *H pylori*-positive family members living together (group Ⅰ) or patients only (group Ⅱ). Nine months after treatment, all patients were assessed for *H pylori* positivity.

RESULTS: There were 70 *H pylori*-positive patients in each group; patients in groups Ⅰ and Ⅱ lived with 175 and 190 *H pylori*-positive relatives, respectively. Age, sex and *H pylori* positivity rate were similar in both groups of relatives. Nine months after 14 d standard triple therapy, *H pylori* positivity was 7.1% in group Ⅰ patients and 38.6% in group Ⅱ patients [$P < 0.01$, OR = 8.61 95% confidence interval (CI): 2.91-22.84].

CONCLUSION: The present results indicate bad environmental hygienic conditions and close intra-familial relationships are important in *H pylori* contamination. These findings indicate all family members of *H pylori*-positive individuals should be assessed for *H pylori* positivity, particularly in developing countries where *H pylori* prevalence is high; they also suggest patients, their spouses and all *H pylori*-positive family members of *H pylori*-positive individuals should be treated for *H pylori* infection.

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Key words: *H pylori*; Gastric adenocarcinoma; Intra-familial infections; Gastric lymphoma; Peptic ulcer disease; Non-ulcer dyspepsia

INTRODUCTION

Almost half of the world's population is infected with *H pylori*[1-3]. The incidence of infection has been reported to be as high as 80% in less developed countries and as low as 20% in Western countries[3]. *H pylori* is involved in the etiology of gastric mucosa-associated lymphoid tissue (MALT)[4] lymphoma and gastric adenocarcinoma, leading the World Health Organization to declare *H pylori* a first degree carcinogen[5-9]. Less invasive diagnostic methods have enabled easier and earlier diagnosis and more widespread use of treatments.

Although *H pylori* infection is thought to occur in childhood, its method of transmission is not known[2,10-13]. Research in highly infected populations has shown the importance of intra-familial infections, bad hygiene and lower socio-economical status in the spread of *H pylori*[2,3,12,14]. However, these routes suggest infection is preventable.

The total time to exposure to *H pylori* has been found to correlate with the development of gastric adenocarcinoma[5-7,15], making diagnosis and eradication of the bacteria crucial. Furthermore, re-infection with *H pylori* may occur after eradication, owing to exposure to untreated individuals, indicating treatment focused on the patient alone will not result in total eradication. We sought to determine whether treatment of *H pylori* should be extended to *H pylori*-positive family members dwelling with the patient, or whether treatment of the patient alone would be sufficient. Treatment extended to the family would theoretically decrease the risk of re-infection.

MATERIALS AND METHODS

A two-armed prospective, randomized study group was composed of *H pylori*-positive patients in our gastroscopy unit with similar socio-economic status and family backgrounds. The sampling number was calculated based on a 20% statistically significant difference (lower limit

Table 1 Relationship of family members to patients

	Sex	Group 1 *n* (%)	Group 2 *n* (%)	*P* value
Spouse	Male	31 (51.7)	31 (51.7)	0.92[2]
	Female	29 (48.3)	30 (49.2)	
1. Child	Male	20 (42.6)	26 (45.6)	0.87[2]
	Female	27 (57.5)	33 (54.4)	
2. Child	Male	18 (66.7)	13 (50.0)	0.29[2]
	Female	9 (33.3)	13 (50.0)	
Mother	Female	16 (59.3)	18 (60.0)	0.99[1]
Father	Male	11 (40.8)	12 (40.0)	
Siblings	Male	3 (50.0)	3 (42.9)	0.99[1]
	Female	3 (50.0)	4 (57.2)	
Grandchild	Male	3 (60.0)	3 (42.9)	0.99[1]
	Female	5 (40.0)	4 (57.1)	

[1]Fisher's absolute χ^2; [2]Yates corrected χ^2.

8%) with type I error = 0.05, type Ⅱ error = 0.02 and power 0.80. The minimum sample size was 67 subjects. Due to possible dropouts, 70 *H pylori*-positive subjects who lived with family members were included in each arm of the study. All patients and family members provided written informed consent. Using a random number table subjects were divided into two equal groups. All family members dwelling with each subject (namely, spouses, children, brothers, sisters, fathers and mothers) were screened for *H pylori* positivity. *H pylori* antigen testing of stools was used to screen children under the age of 14 years and endoscopic biopsy and/or stool test was used to screen all other subjects. In both groups, the ages of the patients as well as their family, sex, relationship to the patient, endoscopic findings, type of *H pylori* screening and positivity were recorded.

Patients randomized to Group I and all *H pylori*-positive family members were treated for *H pylori* infection; patients randomized to Group Ⅱ only were treated, but their family members were not. Patients and their adult relatives received standard triple therapy of clarithromycin, amoxicillin and proton pump inhibitors. Treatment of children and infants was individually handled by pediatric gastroenterologists. Nine months after treatment, all patients in both groups and treated family members in group I were assessed for *H pylori*-positivity using stool antigen tests.

Statistical analysis

A Student's *t* was used for continuous and normative variables, the χ^2 test was used for categorical variables and the McNemar test was used to assess before and after *H pylori* positivity rates. Statistical significance was defined as $P < 0.05$, both directions.

RESULTS

Each group consisted of 70 patients, and there were no drop-outs. The two groups were similar in age and sex ratio ($P = 0.86$, $\chi^2 = 0.02$). The 70 patients in Group I lived with 175 relatives, whereas the 70 patients in Group Ⅱ lived with 190 relatives, all of whom were screened for *H pylori* (Table 1). The number of family members, relationship to each patient, age and sex distribution did not differ significantly between the two groups ($P > 0.05$).

Table 2 Pre-treatment *H pylori* positivity rates in relatives of both groups

	Group 1	*n* (%)	Group 2	*n* (%)	*P* value
Spouse	Positive	46 (76.7)	Positive	48 (78.7)	0.82[1]
	Negative	14 (23.3)	Negative	13 (21.3)	
	Total	60 (100)	Total	61 (100)	
1. Child					
	Positive	23 (48.9)	Positive	28 (47.5)	0.99[1]
	Negative	24 (51.1)	Negative	31 (52.5)	
	Total	47 (100)	Total	59 (100)	
2. Child					
	Positive	12 (44.4)	Positive	11 (42.3)	0.99[1]
	Negative	15 (55.6)	Negative	15 (57.7)	
	Total	27 (100)	Total	26 (100)	
Mother					
	Positive	12 (75.0)	Positive	14 (77.8)	
	Negative	4 (25.0)	Negative	4 (22.2)	
	Total	16 (100)	Total	18 (100)	
Father					
	Positive	8 (72.7)	Positive	9 (75.0)	0.99[1]
	Negative	3 (27.3)	Negative	3 (25.0)	
	Total	11 (100)	Total	12 (100)	
Grandchild	Positive	2 (25.0)	Positive	2 (28.6)	0.99[1]
	Negative	6 (75.0)	Negative	5 (71.4)	
	Total	8 (100)	Total	7 (100)	
Siblings	Positive	3 (50.0)	Positive	4 (57.1)	0.99[1]
	Negative	3 (50.0)	Negative	3 (42.9)	
	Total	6 (100)	Total	7 (100)	

[1]Fisher's exact test.

The most common endoscopic findings in both groups of patients were pan-gastritis and antral gastritis. Diagnostic variables did not differ significantly between the two groups ($P > 0.05$).

Ninety-five family members underwent gastroscopy and biopsy, whereas the remaining family members were assayed for *H pylori* by the stool *H pylori* antigen test. Family members in the two groups who underwent gastroscopy did not differ in number, relationship to patient, gender, age and pathological findings ($P > 0.05$). Of the 95 family members who underwent endoscopy, 75 (78.9%) were spouses. Sixty-three (66.3%) family members presented with various pathologies on gastroscopy, the most common pathologies being antral gastritis (40.9%), pan-gastritis (26.4%) and hiatal hernia (17.7%). The remaining 32 family members had normal gastroscopic findings.

The highest prevalence of *H pylori* positivity was among the spouses of *H pylori*-positive individuals, followed by parents and siblings. Children and grandchildren were among the least likely to be infected. Pretreatment *H pylori* positivity was statistically similar in both groups ($P > 0.05$, Table 2).

All *H pylori*-positive patients in both groups, and *H pylori*-positive family members in Group I, were treated with standard triple therapy for 14 d. Nine months later, 7.1% of Group I patients were *H pylori*-positive, compared with 38.6% of Group Ⅱ patients [$P < 0.001$, OR = 8.61, confidence interval (CI): 2.91-22.84; Table 3].

Table 3 ***H pylori*** **positivity rates after 9 mo in relatives of both groups**

Group 1		Group 2	
	n **(%)**		***n*** **(%)**
Positive	5 (7.2)	Positive	27 (38.6)
Negative	65 (92.8)	Negative	43 (61.4)
Total	70 (100)	Total	70 (100)

$P < 0.001$, OR = 8.61, 95% CI: 2.91-22.84.

Four of the 5 Group I patients (80%) and 24 of the 27 Group Ⅱ patients (88.9%) who remained *H pylori* positive 9 months after initial therapy had *H pylori*-positive spouses at follow-up. The rate of spousal infection in Group Ⅱ was higher at follow-up than before treatment (78.7% *vs* 88.9%). The 65 initially *H pylori*-positive relatives in Group Ⅰ were all *H pylori*-negative at follow-up.

A total of 15 children aged 5 years or less (8 in Group Ⅰ and 7 in Group Ⅱ) were included in the study. Four infants, 2 in each group (26.7%), were initially *H pylori* positive; all in group Ⅰ were *H pylori* negative 9 months after treatment.

DISCUSSION

Although the exact environmental factors contributing to *H pylori* infection are unclear, bodily fluids and personal contact may be key factors in the spread of disease among family members[10-12,14,16]. Due to improved hygiene and environmental conditions, *H pylori* prevalence has decreased significantly in Western countries, but it remains high in developing countries such as Turkey[2,7,17,18]. Lower socio-economic status resulting in more personal contact (for example, sharing sleeping facilities), crowded families and sharing of common living areas with compromised hygiene can also lead to increased dissemination of *H pylori*[2,12,19,20].

Patients who are diagnosed *H pylori* positive should be unquestionably treated. The recommended treatment regimen for *H pylori* eradication consists of 7 to 14 d of triple therapy with clarithromycin, amoxicillin and a proton pump inhibitor[21-24]. Theoretically, in areas and socio-economic groups where intra-familial infection is highly probable, treatment of the patient only may not prevent re-infection over the long term. The present study was designed to investigate this hypothesis.

The most important finding from this study was that statistically significant *H pylori* eradication was achieved in patients whose *H pylori*-positive family members were also treated, compared with those whose *H pylori*-positive family members were not treated, in long-term follow-up (7.1% *vs* 38.6%, group Ⅰ and Ⅱ, respectively). The rate of resistant *H pylori* infection (7.1%) was higher than expected, possibly due to other environmental, life style and hygiene issues outside the family.

H pylori is highly prevalent in the healthy partners of patients with *H pylori* infection. To decrease long-term re-infection rates, family members of *H pylori*-positive patients should be tested and, if positive, treated for *H pylori* infection. Since spouses had the highest *H pylori* positivity rates, our findings suggest that at least the spouse of an *H pylori*-positive patient should be screened and treated if positive. The prevalence of *H pylori* in children aged 5 years or less (6.7%) was also alarming, considering the long-term health problems associated with this infection.

Among the possible routes of *H pylori* infection, the fecal-oral, oro-oral and gastro-oral routes are the most probable. *H pylori* has been cultured in vomit, feces and sputum[2,3,11,12,16,25]. Although intra-familial transmission is a major factor contributing to *H pylori* infection, the relative contributions of close interpersonal contact and genetic similarity are not known[10,26-28]. Spouse-spouse transmission would appear to be an obvious source of infection in adulthood[16,29].

In areas where the risk of contamination is high, re-infection can be prevented by eradication programs[30,31]. Lifestyle changes are recommended, but they are unlikely to occur in the short term. Thus, in such areas, the re-infection rate is high due to contact with the source of *H pylori* infections. Our findings clearly show the necessity of screening and treating all *H pylori*-positive family members to eradicate the source of infection. Larger scale studies, in Turkey and in other developing countries with high *H pylori* infection rates, may determine the routes of infection and other possible methods of prevention.

COMMENTS

Background

The prevalence of *H pylori* infection is higher in developing countries due to higher rates of intra-familial transmission. *H pylori* infection is the causative factor of some gastrointestinal symptoms, such as non-ulcer dyspepsia, peptic ulcer and gastric adenocarcinoma. The whole family besides the *H pylori* positive patient should be given medical treatment.

Research frontiers

Our findings show that new research performed in different population groups are needed to support a new approach to family members in the case of positivity in one family member.

Innovations and breakthroughs

The necessity of medical treatment aiming to eradicate *H pylori* infection in all siblings, especially the spouse, as well as the patient.

Applications

Routine eradication of *H pylori* infection in the whole family and decreasing the prevalence of peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma and gastric cancer.

Peer review

The paper is interesting and the design is correct.

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RAPID COMMUNICATION

Pre-operative hypoalbuminemia is a major risk factor for postoperative complications following rectal cancer surgery

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Abstract

AIM: To determine the relationship between pre-operative hypoalbuminemia and the development of complications following rectal cancer surgery, as well as postoperative bowel function and hospital stay.

METHODS: The medical records of 244 patients undergoing elective oncological resection for rectal adenocarcinoma at Siriraj Hospital during 2003 and 2006 were reviewed. The patients had pre-operative serum albumin assessment. Albumin less than 35 g/L was recognized as hypoalbuminemia. Postoperative outcomes, including mortality, complications, time to first bowel movement, time to first defecation, time to resumption of normal diet and length of hospital stay, were analyzed.

RESULTS: The patients were 139 males (57%) and 105 females (43%) with mean age of 62 years. Fifty-six patients (23%) had hypoalbuminemia. Hypoalbuminemic patients had a significantly larger tumor size and lower body mass index compared with non-hypoalbuminemic patients (5.5 *vs* 4.3 cm; $P < 0.001$ and 21.9 *vs* 23.2 kg/m^2; $P = 0.02$, respectively). Thirty day postoperative mortality was 1.2%. Overall complication rate was 25%. Hypoalbuminemic patients had a significantly higher rate of postoperative complications (37.5% *vs* 21.3%; $P = 0.014$). In univariate analysis, hypoalbuminemia and ASA status were two risk factors for postoperative complications. In multivariate analysis, hypoalbuminemia was the only significant risk factor (odds ratio 2.22, 95% CI 1.17-4.23; $P < 0.015$). Hospitalization in hypoalbuminemic patients was significantly longer than that in non-hypoalbuminemic patients (13 *vs* 10 d, $P = 0.034$), but the parameters of postoperative bowel function were not significantly different between the two groups.

CONCLUSION: Pre-operative hypoalbuminemia is an independent risk factor for postoperative complications following rectal cancer surgery.

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Key words: Hypoalbuminemia; Rectal cancer; Outcomes; Morbidity; Postoperative bowel function

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INTRODUCTION

Malnutrition is a common problem in gastrointestinal cancer patients that adversely affects surgical outcomes[1-3]. There are many tools to assess patients' nutritional status. Serum albumin is a good and simple predictor of surgical risk and has a close correlation with the degree of malnutrition[4]. Hypoalbuminemia is a predictor of delayed recovery of bowel function and is strongly associated with postoperative complications after right hemicolectomy for right-sided colon cancer[5], or following other major gastrointestinal surgery[6-8]. However, there is little information regarding the effect of hypoalbuminemia on the development of postoperative complications after rectal cancer surgery, and the results remain controversial[9-11].

The aims of this study were to determine the relationship between pre-operative hypoalbuminemia and the development of complications following rectal cancer surgery, and to evaluate the effect of hypoalbuminemia on postoperative bowel function and hospital stay.

MATERIALS AND METHODS

Patients and data collection

After obtaining approval from the Institutional Ethics Committee, we carried out a retrospective analysis of patients undergoing elective oncological resection for rectal adenocarcinoma between January 2003 and December 2006 at the Department of Surgery, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand. Rectal cancer was defined as a tumor with the lower edge located within 15 cm of the anal verge, as measured by rigid sigmoidoscopy. Patients were excluded if they had pelvic exenteration, or had had operations for recurrent tumors or acute complicated conditions such as colonic obstruction.

Each patient had a pre-operative serum albumin assessment. Serum albumin less than 35 g/L was recognized as hypoalbuminemia. Every patient underwent pre-operative mechanical bowel preparation. Standard oncological resection of the rectum was performed by either a laparoscopic or open approach. In case of sphincter-saving operation, colorectal anastomosis was performed without colonic pouch formation or protective stoma. In the case of abdominoperineal resection, primary closure of the perineal wound was performed after a pelvic drain had been placed. A nasogastric tube was inserted at the surgeon's discretion.

Routine postoperative care was provided to each patient. Time to first bowel movement (passing flatus) was recorded by nursing staffs. The patients were allowed to have oral fluid if passing flatus. Resumption of a normal diet was decided by surgeons with patients' compliance. Patients were discharged from the hospital if the drain was removed and they had no fever, good appetite and good ambulation. All patients were scheduled for follow-up at 30 d postoperatively.

Data recorded included patient demographic, tumor characteristics, operative details and postoperative outcomes. Patient-related factors included age, gender, American Society of Anesthesiologist (ASA) status, obesity (body mass index over 30 kg/m^2), anemia (pre-operative hematocrit less than 30%), hypoalbuminemia and neoadjuvant chemoradiation. Tumor-related factors included location, size and TNM stage. Operation-related factors included the type of operation and requirement of blood transfusion. Postoperative outcomes, including mortality, complications, time to first bowel movement, time to first defecation, time to resumption of normal diet and length of hospital stay, were analyzed.

Statistical analysis

All data were prepared and analyzed using Statistical Package for the Social Sciences program version 11.0 for Windows (SPSS®, SPSS Inc., Chicago, IL). The Kolmogorov-Smirnov test was used to test for a normal pattern of data distribution. Unpaired *t*-tests were used to compare data between the two groups when they showed a normal distribution pattern. The Mann-Whitney *U* test was used when this was not the case. Pearson's Chi-square test and Fisher's exact test were used when the data were quantitative. Stepwise regression analysis was used for multivariate analysis in order to determine any confounding factors. A *P*-value of less than 0.05 was considered statistically significant.

Table 1 Demographic data on non-hypoalbuminemic and hypoalbuminemic patients

	Non-hypoalbuminemic (*n* = 188)	Hypoalbuminemic (*n* = 56)	*P* value
Age (yr)	61.4 ± 11.9	64.5 ± 13.5	0.100
Male	111 (59)	28 (50)	0.230
BMI (kg/m^2)	23.2 ± 3.8	21.9 ± 3.4[a]	0.021
Tumor size (cm)	4.3 ± 1.7	5.5 ± 2.3[b]	< 0.001
TNM stage			0.310
Ⅰ	48 (26)	8 (14)	
Ⅱ	43 (23)	14 (25)	
Ⅲ	94 (50)	34 (61)	
Ⅳ	3 (1)	0 (0)	
Type of anesthesia			0.860
GA alone	73 (39)	21 (38)	
GA + EB	115 (61)	35 (62)	

GA: General anesthesia; EB: Epidural block. Values were given as number (percentage) or mean ± SD. [a]$P \leqslant 0.05$, [b]$P \leqslant 0.01$ *vs* Non-hypoalbuminemic.

RESULTS

Two hundred and forty four patients were studied. There were 139 males (57%) and 105 females (43%) with a mean age of 62 years (range 23-87). Fifty-six patients (23%) had hypoalbuminemia. Patients with hypoalbuminemia had a significantly larger size of the tumors and lower body mass index (BMI), compared with those without hypoalbuminemia (5.5 *vs* 4.3 cm; $P < 0.001$ and 21.9 *vs* 23.2 kg/m^2; $P = 0.02$, respectively). Gender and tumor staging were not statistically different between the two groups (Table 1).

Thirty day postoperative mortality was 1.2% (3 patients died; one massive pelvic hemorrhage, one perioperative myocardial infarction, and one anastomotic leakage with respiratory failure). Overall complication rate was 25%. Infectious complications were diagnosed in 59 patients (24%); 42 surgical site infections, 13 anastomotic leakage, 2 urinary tract infection and 2 pneumonia. Two patients (1%) developed noninfectious complications during hospitalization (one acute respiratory failure and one perioperative myocardial infarction). Hypoalbuminemic patients had a significantly higher rate of postoperative complication (37.5% *vs* 21.3%, $P = 0.014$).

Univariate analysis revealed hypoalbuminemia and ASA status as two risk factors for the development of postoperative complications (Table 2). In multivariate analysis, hypoalbuminemia was the only significant risk factor for postoperative complications (odds ratio 2.22, 95% CI 1.17-4.23; $P < 0.015$). Length of hospital stay in hypoalbuminemic patients was significantly longer than that in non-hypoalbuminemic patients (13.0 *vs* 10.4 d, $P = 0.034$), but time to first bowel movement, time to first defecation and time to resumption of normal diet were not significantly different between the two groups (Table 3).

DISCUSSION

Patients with rectal cancer are at risk of malnutrition due to cancer-induced higher metabolism, dietary intake reduction, and cancer cachexia[12-14]. Tumor necrosis factor-alpha is considered to be the main mediator of cancer cachexia

Table 2 The relationship between the clinical characteristics of the patients and the development of postoperative complications analyzed using a univariate model

Variables	Category	*n*	Complication rate, *n* (%)	*P* value
Age	⩾ 60	102	26 (25.5)	0.880
	< 60	142	35 (24.6)	
Gender	Male	139	38 (27.3)	0.330
	Female	105	23 (21.9)	
Obesity	Yes	10	3 (30.0)	0.710
	No	234	58 (24.8)	
ASA status	Ⅰ	65	11 (16.9)	0.041
	Ⅱ	162	42 (25.9)	
	Ⅲ	17	8 (47.1)[a]	
Anemia	Yes	18	6 (33.3)	0.390
	No	226	55 (24.3)	
Hypoalbuminemia	Yes	56	21 (37.5)	0.014
	No	188	40 (21.3)[a]	
Neoadjuvant therapy	Yes	23	5 (21.7)	0.900
	No	221	56 (25.3)	
Tumor size	⩾ 5 cm	105	27 (25.7)	0.820
	< 5 cm	139	34 (24.4)	
Rectal tumor location	Upper	56	14 (25.0)	0.430
	Middle	134	30 (22.3)	
	Lower	54	17 (31.4)	
Sphincter-saving procedure	Yes	179	44 (24.6)	0.800
	No	65	17 (26.2)	
Operation time	> 4 h	87	24 (27.6)	0.490
	< 4 h	157	37 (23.6)	
Blood transfusion	Yes	32	9 (28.1)	0.660
	No	212	52 (24.5)	

[a]$P \leqslant 0.05$.

as it is responsible for different metabolic alterations and leads to impairment of hepatic protein synthesis[15]. Cancer patients also have increased whole protein turnover and subsequent body nitrogen loss[16]. Albumin has a long half-life of 20 d, so that metabolic effects on its concentration reflect prolonged malnourishment, as in cancer patients. Hypoalbuminemia is widely accepted to be a good indicator for malnutrition in many national cancer studies[7,11,17]. It is remarkable that tumor size, but not the stage, is relevant to hypoalbuminemia. The clear explanation of this association remains unknown. It is possible that large tumors cause more gastrointestinal symptoms, leading to poor intake and partial gut obstruction.

In this present study, univariate analysis showed that hypoalbuminemia and ASA status were significant risk factors for postoperative complications following rectal cancer surgery. However, hypoalbuminemia was the only independent factor for postoperative morbidity after multivariate analysis. Our finding is consistent with those of two large population-based prospective studies in France[7] and the United States[11]. Gibbs *et al*[11] reported that a decrease in serum albumin from concentrations greater than 46 g/L to less than 21 g/L was associated with an exponential increase in mortality rates from less than 1% to 29% and in morbidity rates from 10% to 65% following non-cardiac surgery. Moreover, hypoalbuminemia was a better predictor of some types of morbidity, especially sepsis and major infections. Ryan *et al*[18] showed decreased serum albumin concentration on the first postoperative day was also an independent predictor of poor surgical outcome following gastrointestinal cancer surgery.

Table 3 Univariate analysis of clinical outcomes (mean ± SD)

	Non-hypoalbuminemic (*n* = 188)	Hypoalbuminemic (*n* = 56)	*P* value
Postoperative complication (%)	21.3	37.5[a]	0.014
Time to first bowel movement (h)	2.3 ± 1.4	2.7 ± 1.6	0.090
Time to first defecation (d)	4.5 ± 1.7	4.6 ± 2.5	0.730
Time to resumption of normal diet (d)	4.8 ± 2.7	5.4 ± 2.9	0.180
Length of hospital stay (d)	10.4 ± 8.0	13.0 ± 8.9[a]	0.034

[a]$P \leqslant 0.05$.

Hypoalbuminemia was associated with poor tissue healing, decreased collagen synthesis in the surgical wounds or at the anastomosis[19-21], and impairment of immune responses, such as macrophage activation and granuloma formation[22,23]. Therefore, in hypoalbuminemic patients, wound infection, remote infections such as pneumonia, and anastomotic leakage, were commonly found.

According to the present study, a tendency towards delayed recovery of postoperative bowel function has also been found in hypoalbuminemic patients, although this effect did not reach statistical significance. This may be because such patients develop more complications, including intra-abdominal sepsis, and because lower serum albumin causes swelling and edema of the intestine[24], resulting in paralytic ileus. Clearly, there would be other factors contributing to postoperative bowel function, such as narcotics administration and electrolyte status[25-27]. Prolonged hospitalization in hypoalbuminemic patients may be the result of higher rates of postoperative complication and delayed bowel recovery.

With regard to the treatment of pre-existing hypoalbuminemia, no published studies have been able to demonstrate any advantage of albumin infusion on morbidity and mortality[28,29]. This may be because of insufficient albumin replacement or increased leakage of synthetic albumin into the extravascular spaces. However, a recent prospective randomized study reported the administration of a supplemented diet before and after surgery was beneficial to outcomes in malnourished patients with gastrointestinal cancer[30].

In conclusion, pre-operative hypoalbuminemia is an independent risk factor for postoperative complications following rectal cancer surgery. Hypoalbuminemic patients also have a nonstatistically significant tendency to delayed recovery of postoperative bowel function. Low serum albumin may be used as a simple and low-cost prognostic tool to predict the risk of adverse surgical outcomes.

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COMMENTS

Background

Malnutrition is a common problem in gastrointestinal cancer patients that adversely affects surgical outcomes. There are many tools to assess patients' nutritional status. Serum albumin is a good and simple predictor of surgical risk and has a close correlation with the degree of malnutrition. Hypoalbuminemia is a predictor of delayed recovery of bowel function and is strongly associated with postoperative complications in colon cancer surgery.

Research frontiers

There is little information regarding the effect of hypoalbuminemia on the development of postoperative complications after rectal cancer surgery, and the results remain controversial.

Innovations and breakthroughs

The present study clearly demonstrates hypoalbuminemia is associated with an increased rate of complications, and has a negative impact on postoperative recovery following rectal cancer surgery.

Applications

Pre-operative assessment of patients with rectal cancer may routinely include serum albumin levels. Patients with pre-operative hypoalbuminemia should be informed about the higher rate of complications after rectal cancer surgery. Every effort to improve patients' pre-operative malnourishment without delayed surgical treatment should be encouraged. Further research might focus on the effect of hypoalbuminemia on long-term surgical outcomes and cancer survival.

Terminology

Pre-operative hypoalbuminemia is an independent risk factor for postoperative complications following rectal cancer surgery.

Peer review

This is an interesting paper showing that hypoalbuminemia will have an impact on postoperative recovery and complication. It is well known that malnourished patients do worse than those with a good nutrition status in the postoperative course. It is well written, the data is excellent and references timely.

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RAPID COMMUNICATION

P wave dispersion is prolonged in patients with Wilson's disease

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Abstract

AIM: To investigate the P wave dispersion as a non-invasive marker of intra-atrial conduction disturbances in patients with Wilson's disease.

METHODS: We compared Wilson's disease patients (n = 18) with age matched healthy subjects (n = 15) as controls. The diagnosis was based on clinical symptoms, laboratory tests (ceruloplasmin, urinary and hepatic copper concentrations). P wave dispersion, a measurement of the heterogeneity of atrial depolarization, was measured as the difference between the duration of the longest and the shortest P-waves in 12 lead electrocardiography.

RESULTS: All the patients were asymptomatic on cardiological examination and have sinusal rhythm in electrocardiography. Left ventricular and left atrial diameters, left ventricular ejection fraction and left ventricular mass index were similar in both groups. The Wilson's disease patients had a significantly higher P wave dispersion compared with the controls (44.7 ± 5.8 *vs* 25.7 ± 2.5, P < 0.01).

CONCLUSION: There was an increase in P wave dispersion in cardiologically asymptomatic Wilson's disease patients which probably represents an early stage of cardiac involvement.

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Key words: Wilson's disease; Electrocardiography; P wave duration; P wave dispersion; Atrial depolarization

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INTRODUCTION

The persistence of P-wave duration is an accepted indicator of a disturbance in the interatrial conduction[1,2]. P wave dispersion (PWD) constitutes an important contribution to the field of noninvasive electrocardiology and is defined as the difference between the longest and shortest P wave duration recorded from surface electrocardiogram (ECG) leads. PWD has been thoroughly examined in a number of diseases including hypertension, coronary artery disease, coronary artery bypass surgery, and paroxysmal atrial fibrillation (AF)[3-7]. Therefore, it has been suggested that PWD can be used to diagnose patients with a high risk of AF[8-13]. Wilson's disease is a severe genetic metabolic disorder, which is associated with intracellular copper overload and multiple organ involvement. Cardiac manifestations in Wilson's disease include arrhythmias, cardiomyopathy, cardiac death, and autonomic dysfunction[14]. To our knowledge, no previous studies have compared P wave duration and PWD of Wilson's disease patients to healthy controls. The aim of this study was to investigate the PWD as a non-invasive marker of intra-atrial conduction disturbance in patients with Wilson's disease.

MATERIALS AND METHODS

Subjects

Eighteen cardiologically asymptomatic patients with Wilson's disease and 15 healthy subjects were included in the study. We excluded patients with previous acute myocardial infarction, thyroid dysfunction, uncontrolled diabetes mellitus, chronic renal disease, valvular heart disease, cardiomyopathy, chronic obstructive pulmonary disease, systemic

or pulmonary hypertension and alcohol abuse. All of the patients were in sinus rhythm and none were taking medications like antiarrhythmics, tricyclic antidepressants, antihistaminic and antipsychotics. The diagnosis of Wilson's disease was established based on the clinical manifestations, family history of neuropsychiatric manifestations, jaundice, and premature death attributable to Wilson's disease, evidence of Kayser-Fleischer rings on slit lamp examination, low serum copper and ceruloplasmin assay, and increased 24-h urinary excretion of copper. Radiologic investigations included a cranial computed tomography (CT) scan with or without iodinated contrast and/or magnetic resonance imaging (MRI) and X-rays of long bones, pelvis, and chest to evaluate for skeletal abnormalities[15]. The diagnosis is confirmed by the liver biopsy and quantitative liver copper assay[16].

Echocardiographic measurements

In all subjects, two-dimensional, M-mode pulsed and color flow Doppler echocardiographic examinations (Vivid 7 Dimension, GE, Horten, Norway) were performed by the same examiner. Internal left ventricular (LV) end-diastolic and end-systolic diameters and interventricular septal and posterior wall thickness at end-diastole, and left atrial dimension were measured from parasternal long axis window in M-mode echocardiography[17]. The ejection fraction of the left ventricle was obtained using modified Simpson's method[18].

Conventional Doppler echocardiography

Early diastolic wave peak velocity (E), late diastolic wave peak velocity (A), early to late velocities (E/A) ratio and E wave deceleration time of left ventricular inflow velocities were measured by pulse wave Doppler placing the sample volume in-between the tips of the mitral valve leaflets in apical four-chamber window. Isovolumic relaxation time (IVRT) was obtained from the apical-five-chamber view by placing the sample volume between the tip of the mitral anterior leaflet and left ventricle outflow tract.

Electrocardiography

Twelve-lead ECGs of all patients at rest, with 1 mV/cm amplitude and 50 mm/s rate, were obtained. The P-wave onset was defined as the first atrial deflection from the isoelectric line and the offset was the return of the atrial signal to baseline. Patients whose measurements could be performed in at least 8 derivations were included in the study. In all patients, derivations were excluded if the beginning or the ending of the P wave could not be clearly identified.

Maximum P wave duration (P_{max}) is defined as the longest and minimum P wave duration (P_{min}) is defined as the shortest P wave duration. PWD defined as difference between P_{max} and P_{min}. All the measurements were repeated three times and average values were calculated for each of electrocardiographic parameter. All of the measurements were performed using the same experienced investigators blind to the subject's clinical status. Intra-observer and inter-observer variability was assessed in a random sample of 15 ECG (10 from patients who have Wilson's disease and 5 from control subjects) by a second investigator. The study was approved by the local ethics committee of our institution, and all patients gave written informed consent.

Table 1 The demographic and clinical characteristics of the study population

	Wilson's patients (n = 18)	Control group (n = 15)	P
Age (yr)	49 ± 26	44 ± 11	0.424
Left atrium (cm)	3.1 ± 0.4	3.2 ± 0.6	0.240
Left ventricular end diastolic diameter (cm)	4.5 ± 0.3	4.7 ± 0.4	0.316
Left ventricular end systolic diameter (cm)	2.8 ± 0.3	3.0 ± 0.3	0.320
Interventricular septum thickness (cm)	0.8 ± 0.1	0.9 ± 0.1	0.435
Posterior wall thickness (cm)	0.9 ± 0.1	0.8 ± 0.1	0.349
Ejection fraction (%)	66.6 ± 6.6	64.6 ± 6.1	0.335
E (cm/s)	93.7 ± 14.3	88.2 ± 12.5	0.323
A (cm/s)	68.8 ± 14.6	75.2 ± 25.1	0.303
E/A	1.3 ± 0.4	0.8 ± 0.2	0.090
EDT (ms)	177 ± 53.2	167 ± 34.2	0.213
IVRT (ms)	68.4 ± 12.0	72.2 ± 22.4	0.275
P wave dispersion (ms)	44.7 ± 5.8	25.7 ± 2.5	0.007
Minimum P wave duration (ms)	65 ± 12	74 ± 10	0.239
Maximum P wave duration (ms)	109 ± 8	102 ± 10	0.031

Statistical analysis

The SPSS statistical software package (11.0) was used to perform all statistical calculations. Number of sample is expressed as n, continuous variables were expressed as mean ± SD, and categorical variables as percentages. Pearson correlations were used to compare the association between indexes. Categorical variables were compared by Pearson Chi-square test. Comparisons of continuous variables between two groups have been performed by means of unpaired Student's t test. For all tests, $P < 0.05$ was considered statistically significant.

RESULTS

The study included 18 patients (age: 49 ± 26 years, range 10-49 years) with Wilson's disease and 15 healthy controls (age: 44 ± 11 years, range 25-50 years). In Wilson's disease patients, the patients' age at the diagnosis was 42 ± 18 years (range, 2.5-42 years), the mean disease duration was 9.6 ± 7 years (range, 1-29 years). Serum copper, ceruloplasmin and urinary copper excretion were 1670 ± 800 μg/L, 300 ± 120 mg/L, 167 ± 80 μg/dL, respectively. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), hemoglobin, total cholesterol, low density lipoprotein, high density lipoprotein, and triglyceride levels were 53.2 ± 42 IU/L, 45.6 ± 35 IU/L, 138 ± 18 mg/L, 131 ± 37 mg/dL, 50 ± 31 mg/dL, 66 ± 23 mg/dL, and 95 ± 16 mg/dL respectively.

The demographic and clinical characteristics of the Wilson's patients and the controls were shown in Table 1. There was no significant difference between the two groups in regard to gender, age, body mass index (BMI), heart rate or blood pressure. All Wilson's patients were treated with copper chelation therapy. Seventeen of the

Wilson's patients have been treated with D -penisillamine (0.75-1 g p.o., t.i.d.) and one patient was switched to trientine (750 mg, p.o. t.i.d.) of because drug related thrombocytopenia.

P_{max} and PWD were significantly higher in Wilson's patients than controls. However, there was no significant difference in P_{min} between the two groups (Table 1; A = Transmitral late diastolic peak velocity, E = Transmitral early diastolic peak velocity, EDT = E wave deceleration time, IVRT = Isovolumic relaxation time).

PWD was considerably correlated only with the age at diagnosis (r = -0.606, P = 0.048), serum copper level (r = -0.801, P = 0.009) and mitral E wave velocity (r = -0.724, P = 0.027) in patients with Wilson's disease. We were not able to find any statistically significant correlation between PWD and other clinical and echocardiographic parameters.

The intra-observer standard deviation for P_{max}, P_{min} and PWD were 10.3, 6.5, and 11 ms, respectively, and the corresponding intra-observer variability was 0.05%, 0.06%, and 0.2%, respectively. The inter-observer standard deviation for P_{max}, P_{min} and PWD were 10.5, 11.0, and 13.9 ms, respectively, and the corresponding inter-observer variability was 0.04%, 0.2%, and 0.5%, respectively. The percentage difference in P_{max}, P_{min} and PWD were 2.4%, 3.5%, and 6% within observers and 3%, 5.2%, and 5.9% between observers.

DISCUSSION

Wilson's disease is a severe genetic multisystem disorder associated with intracellular copper storage. Wilson's disease is characterized by an inadequate excretion of absorbed dietary copper *via* bile resulting in the accumulation of toxic amounts of copper in the liver and other organs. It is inherited as a rare autosomal recessive condition with an incidence of one in 40 000 live births in most populations, and with a calculated carrier frequency in the general population of one in 90[19,20]. Copper is an essential micronutrient but ionic copper is toxic. The toxic effects are thought to be mediated by the generation of reactive oxygen free radical species in Wilson's patients[19]. Cardiac involvement in Wilson's disease has rarely been recognized. Cardiac manifestations in Wilson's disease include arrhythmias, cardiomyopathy, cardiac death, and autonomic dysfunction[14,19]. Electrocardiographic abnormalities occurred in 34 percent, including left ventricular hypertrophy, biventricular hypertrophy, early re-polarization, ST depression and T inversion, premature atrial or ventricular contractions, atrial fibrillation, sino-atrial block and Mobitz type 1 atrio-ventricular block. Asymptomatic orthostatic hypotension, an abnormal response to the Valsalva maneuver, ventricular fibrillation, and dilated cardiomyopathy can be occurred in Wilson's disease[14].

The major pathological findings of the myocardium in Wilson's disease included the presence of interstitial and replacement myocardial fibrosis, intra-myocardial small vessel disease, focal myocarditis and cardiac hypertrophy, AV nodal degeneration and occlusive atherosclerosis in early ages[21]. These alterations are non-specific, but they are similar to those observed in other cardiomyopathies[21,22]. Their existence in a relatively young group of patients without other significant etiology for the development of heart disease, suggests the possibility of a direct relationship between Wilson's disease and cardiac degeneration[20].

Therefore, we investigated PWD in patients with Wilson's disease patients. PWD is a new electrocardiographic marker that has been associated with the heterogeneous and discontinuous propagation of sinus impulses. Furthermore, the correlation between the presence of intra-atrial conduction abnormalities and the induction of paroxysmal AF has been well documented[8-10,23,24]. Prolonged P wave duration and increased PWD have been reported to carry an increased risk for atrial fibrillation[5-7,17]. Therefore, it has been suggested that PWD can be used to diagnose patients with a high risk for developing AF[8-10]. To our knowledge, this is the first study that has investigated the P wave duration and PWD changes in Wilson's disease patients. This study shows that P_{max} and PWD are higher in Wilson's disease patients than control subjects. These results suggest that Wilson's disease patients may be under the risk for atrial fibrillation. This study also shows that PWD was correlated with mitral E wave velocity which is a parameter of left ventricular diastolic function and the serum copper level. Previously, it has been reported that PWD was associated with diastolic dysfunction and coronary artery disease[3,25,26].

The precise mechanism of arrhythmias seen in Wilson's disease patients is not clear. A recent report Kaduk *et al*[27] describing cardiomyopathy in Wilson's disease, suggested that mitochondrial alterations were the consequence of the accumulation of myocardial copper These alterations are non-specific, and in some cases of limited severity; however, in previous study it was concluded that cardiac degeneration might have contributed to the death of Wilson's patients[27]. PWD may well be associated with autonomic dysfunction in patients with Wilson's disease[27]. Dysautonomia, often subclinical, is only one of the many features of Wilson's disease[28]. A central, rather than peripheral mechanism is hypothesized. Sympathetic and parasympathetic arms are affected equally. The abnormality is independent of involvement of the liver and the duration and severity of Wilson's disease[29].

Previous experiments showed that electrical remodeling of atrial myocardium could be induced by autonomic nervous transmitters and suggested that autonomic nerve activity was an important factor to promote AF episodes[30]. This study population is relatively small and therefore our results should not be extrapolated to all Wilson's patients. Further studies are necessary to investigate the frequency of atrial arrhythmias by rhythm holter in patients with Wilson's disease, who have or do not have high PWD.

Furthermore, one of the relative limitations of our study was that we could not use the method of digital recording and storing of 12-lead electrocardiograms with onscreen measurement of P waves duration which provides the most accurate method for PWD calculation[31]. Secondly, the ability of P wave duration and PWD to predict future atrial fibrillation episodes was not checked in present study, since the patients included in this work were not followed-up. But, it has been well documented previously that prolonged P wave duration and increased PWD carry

an increased risk for atrial fibrillation[8-10,23,24] and therefore, it has been suggested that PWD can be used to diagnose patients with a high risk for developing AF[8-10].

Consequently, involvement of the heart may be seen in patients with Wilson's disease even in the absence of clinical cardiac manifestations. In this study, P_{max} and PWD were found to be higher in patients with Wilson's disease than healthy control subjects. Therefore, the patients with Wilson's disease who have increased PWD should be closely followed for atrial arrhythmias.

COMMENTS

Background

Wilson's disease is a rare severe genetic metabolic disorder associated with intracellular copper overload and related complications. Cardiac manifestations in Wilson's disease include arrhythmias, cardiomyopathy, cardiac death, and autonomic dysfunction. P wave dispersion, a measurement of the heterogeneity of atrial depolarization.

Research frontiers

No previous studies have compared P wave duration and P wave dispersion (PWD) of Wilson's disease patients to healthy controls. The aim of this study was to investigate the PWD as a non-invasive marker of intra-atrial conduction disturbance in patients with Wilson's disease.

Innovations and breakthroughs

Prolonged P wave duration and increased PWD have been reported to carry an increased risk for atrial fibrillation. Therefore, it has been suggested that PWD can be used to diagnose patients with a high risk for developing AF. In previous reports researchers showed that electrical remodeling of atrial myocardium could be induced by autonomic nervous transmitters and suggested that autonomic nerve activity was an important factor to promote AF episodes. This study also shows that PWD was correlated with mitral E wave velocity which is a parameter of left ventricular diastolic function and the serum copper level. Previously, it has been reported that PWD was associated with diastolic dysfunction and coronary artery disease.

Applications

Prolonged P wave duration and increased PWD carry an increased risk for AF. Therefore, it has been suggested that PWD can be used to diagnose patients with a high risk for developing AF. This study shows that Pmax and PWD are higher in Wilson's disease patients than control subjects. These results suggest that Wilson's disease patients may be under the risk for atrial fibrillation.

Terminology

Wilson's disease is a severe genetic metabolic disorder associated with intracellular copper overload and multiple organ involvement. P wave dispersion was measured as the difference between the duration of the longest and the shortest P-waves in 12 lead electrocardiography.

Peer review

This report is original and has to be considered interesting. The authors aimed to investigate the P wave dispersion as a non-invasive marker of intra-atrial conduction disturbances in patients with Wilson's disease. This rather small but homogenous patient population showed promising results with the application of PWD at such a rare genetic disorder.

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RAPID COMMUNICATION

Screening serum hepatocellular carcinoma-associated proteins by SELDI-based protein spectrum analysis

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Abstract

AIM: To find out potential serum hepatocellular carcinoma (HCC)-associated proteins with low molecular weight and low abundance by SELDI-based serum protein spectra analysis, that will have much application in the diagnosis or differentiated diagnosis of HCC, as well as giving a better understanding of the mechanism of hepato-carcinogenesis.

METHODS: Total serum samples were collected with informed consent from 81 HCC patients with HBV(+)/cirrhosis(+), 36 cirrhosis patients and 43 chronic hepatitis B patients. Serum protein fingerprint profiles were first generated by selected WCX2 protein chip capture integrating with SELDI-TOF-MS, then normalized and aligned by Ciphergen SELDI Software 3.1.1 with Biomarker Wizard. Comparative analysis of the intensity of corresponding protein fingerprint peaks in normalized protein spectra, some protein peaks with significant difference between HCC and cirrhosis or chronic hepatitis B were found.

RESULTS: One hundred and twenty-eight serum protein peaks between 2000 and 30 000Da were identified under the condition of signal-to-noise > 5 and minimum threshold for cluster > 20%. Eighty-seven of these proteins were showed significant differences in intensity between HCC and cirrhosis ($P < 0.05$). Of the above differential proteins, 45 proteins had changes greater than two-fold, including 15 upregulated proteins and 30 downregulated proteins in HCC serum. Between HCC and chronic hepatitis B, 9 of 52 differential proteins ($P < 0.05$) had intensities of more than two-fold, including 2 upregulated proteins and 7 downregulated proteins in HCC serum. Between cirrhosis and chronic hepatitis B, 28 of 79 significant differential proteins ($P < 0.05$) changes greater than two-fold in intensity, including 17 upregulated proteins and 11 downregulated proteins in cirrhosis serum. For the analysis of these leading differential proteins in subtraction difference mode among three diseases, the five common downregulated proteins in HCC serum (M/Z 2870, 3941, 2688, 3165, 5483) and two common upregulated proteins (M/Z 3588, 2017) in HCC and cirrhosis serum were screened.

CONCLUSION: Because the interference of unspecific secreted proteins from hepatitis B and cirrhosis could be eliminated partly in HCC serum under subtraction difference analysis, these seven common differential proteins have the obvious advantage of specificity for evaluating the pathological state of HCC and might become novel candidate biomarkers in the diagnosis of HCC.

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Key words: Hepatocellular carcinoma; SELDI-TOF-MS

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide, with the highest incidence being in east and southeast Asia and sub-Saharan Africa, and the estimated annual number of cases is > 500 000[1,2]. In recent years, an increasing incidence and mortality rate of HCC have also been observed in western industrialized countries[3,4]. Most studies on the etiology of HCC suggest that risk factors such as HBV/HCV, cirrhosis, dietary aflatoxin B1 (AFB1) intake, and alcohol abuse are responsible for almost all HCC development. Both HBV

and HCV are attributed to > 80% of HCC development worldwide[5]. In high incidence areas of China, > 90% of HCC cases are reported to have an HBV background[6]. Liver cirrhosis, regardless of its etiology, is recognized as the most powerful independent risk factor for HCC, and about 55%-85% of cases finally develop into HCC with poor prognosis[7,8]. On the other hand, the other two known risk factors (AFB1 and alcohol) only appear as synergistic factors with pre-existing chronic hepatitis or cirrhosis during liver carcinogenesis[9,10]. Although much is known about the effects of the above etiological agents on HCC development, the detailed pathological mechanisms of HCC remain to be unequivocally defined, mainly due to its complex multigene and multifactorial involvement, as well as strong associations with hepatitis or cirrhosis.

Presently, early surgical interventions, including hepatectomy or percutaneous radio frequency ablation, are still the best hope for a clinical cure of HCC. Unfortunately, many cases of HCC exhibit intrahepatic and distant metastasis at the time of diagnosis, which miss the optimal opportunity for surgical resection. This indirectly indicates no ideal tumor markers can be recommended for early detection or differential diagnosis of HCC. Thus, discovering more HCC-associated proteins and identifying a single or a set of reliable, non-invasive new biomarkers is one of the best options for improving the diagnostic efficiency of HCC.

Alteration of serum protein profiles can effectively reflect the pathological states of liver injury. It seem much more challengeable to screen specific HCC-associated proteins in serum of HCC patients with the background of HBV/HCV or cirrhosis because hepatitis and cirrhosis can also cause great changes in the serum protein profile. Moreover, separation and identification of disease-associated proteins is laborious and difficult, relying on low-technology approaches and screening strategies. While some serum tumor markers, including enzymes (GGT (gamma-glutamyl transferase), AFU (alpha-1-fucosidase), DCP (des-gamma-carboxyprothrombin), and oncofetal and glycoprotein antigens [AFP (alpha fetoprotein), AFP-L3 (alpha fetoprotein-L3), GPC3 (glypican-3)], have been widely applied to the detection of HCC, the diagnostic accuracy for HCC is still unsatisfactory, especially in early and differential diagnosis. Even though AFP is a well-known biomarker for HCC, its sensitivity has been reported at 39%-64% and its specificity as 76%-91%, it has been shown to be particularly useful in detecting HCC with non-viral etiology[11,12]. Unlike conventional serological biomarkers with high molecular weight (MW) and high abundance, a lot of uncharacterized serum proteins with low MW and low abundance, which promise to contain important disease information, have attracted much attention in the diagnosis of HCC[13,14]. A promising high-throughput proteomics approach, on-chip protein fractionation coupled to SELDI-TOF-MS (surface enhanced laser desorption ionization-time of flight-mass spectrometry), provides a novel pathway for rapid screening and identification of specific tumor markers with low MW in the proteomes of body fluids. To date, a number of single or combined SELDI spectra peaks have been successfully identified by means of this technology and used to discriminate differential diseases as disease related biomarkers[11,15,16].

In the present study, by comparative analysis of SELDI serum spectra between HCC with HBV(+)/cirrhosis(+) and cirrhosis or HBV, using Ciphergen SELDI Software 3.1.1 with Biomarker Wizard and subtraction difference analysis, some potential serum common HCC-associated proteins with low MW were discovered, which might be new candidate biomarkers for HCC diagnosis.

MATERIALS AND METHODS

Study population and sample preparation

With patient consent, serum samples were obtained from 81 HCC patients with HBV/cirrhosis from the Liver Cancer Institute of Zhongshan Hospital, Fudan University, China. All HCC patients were diagnosed according to combined clinical criteria, including imaging data and serum tumor markers, and further confirmed by histopathological analysis. Samples from 36 patients with liver cirrhosis were kindly provided by the Department of Infection of Shanghai East Hospital, Shanghai, China. Diagnosis of liver cirrhosis was mainly dependent on clinical history, physical examination, laboratory findings, ultrasonography and/or computed tomography, with or without liver biopsy. Serum samples of 43 HBV-infected patients were collected based on the presence of HBsAg (hepatitis B surface antigen), HBeAg (hepatitis B e antigen) and HBV DNA, from Shandong Provincial Hospital, China. All serum samples were separated and divided into 10-μL aliquots, then stored at -80℃ until assayed.

Ciphergen protein chip SELDI-TOF-MS analysis

Compared with other protein chips (anionic, copper mental binding and hydrophobic), the WCX2 protein chip (Ciphergen Biosystems, Fremont, CA, USA) was selected for further serum differential protein spectrum analysis because it generates the best serum profile with the largest number and resolution of protein fingerprint peaks. Serum samples were first diluted 1:2 with U9 sample buffer containing 9 mol/L urea, 2% CHAPS, 50mmol/L Tris/HCl and 1% DTT (pH 9.0, Sigma, USA). Subsequently, 108 μL WCX2 binding buffer (50 mmol/L sodium acetate, pH 4.0; Sigma) was mixed with the above samples homogeneously. One hundred microliters of diluted serum samples was then applied to each spot on pre-equilibrated WCX2 protein chips, packed in a processor for 60 min incubation with shaking. After two washes with WCX2 binding buffer and one quick rinse with HPLC grade water, 1 μL of a saturated solution of EAM sinapinic acid (Ciphergen Biosystems) in 50% acetonitrile and 0.5% trifluoroacetic acid (Sigma) was loaded on each spot and air-dried for MS detection. Mass accuracy was calibrated externally through the use of the All-In-One peptide molecular mass standard (Ciphergen Biosystems). The protein chips were read using the PBSII MS system (Ciphergen Biosystems). Each spot on protein chips was scanned with a laser intensity of 185 and a detector sensitivity of 8, and data acquisition parameters were set as 22. delta to 4. transients per to 7 ending position to 82 by SELDI Quantitation. Assessment of the reproducibility

Table 1 Common significant differential protein peaks between HCC with cirrhosis(+)/HBV(+) and HBV or cirrhosis

Peak M/Z	*P*	Mean HBV	Mean HCC	HBV/ HCC	Peak M/Z	*P*	Mean C	Mean HCC	C/ HCC	Peak M/Z	*P*	Mean C	Mean HBV	C/ HBV
2870	0	2.4084	0.5602	4.2992	2870	0	4.1231	0.5602	7.3599	2870	0.005179992	4.1231	2.4084	1.7119
3941	0	9.081	3.3872	2.681	3941	1.01E-08	8.5539	3.3872	2.5253					
2688	1.00E-10	1.5411	0.5736	2.6869	2688	0	1.489	0.5736	2.5961					
3165	7.51E-08	1.8627	0.9297	2.0036	3165	7.00E-10	2.7519	0.9297	2.9601					
5483	0.000000168	1.6028	0.7756	2.0665	5483	1.54E-08	1.9195	0.7756	2.4749					
3588	0.00012821	0.3225	0.7492	0.4305						3588	0.00006427	0.9533	0.3225	2.9555
2017	0.017271682	0.2451	0.5364	0.4569	2017	0.000903778	0.6541	0.5364	1.2196	2017	1.89E-06	0.6541	0.2451	2.6691

of the SELDI system was the same as in our previous study[17]. The intra- and inter-assay CV for intensity and m/z in this SELDI system were 17.46% and 0.024%, and 17.74% and 0.0237%, respectively.

Bioinformatics and subtraction difference analysis

Protein spectra were automatically generated after all raw data were collected. All spectra were first normalized to a total ion current with an m/z of 2000 and 30 000 and then aligned by baseline subtraction, mass accuracy calibration using Ciphergen SELDI Software 3.1.1 with Biomarker Wizard. Protein peaks ranging from 2 to 30 kDa were autodetected in term of an S/N > 5 and a minimum threshold for cluster > 20%, and were further checked and labeled to minimize mismatched peaks. The m/z value within the 0.3% mass accuracy windows was defined to be identical between replicates. By comparative analysis of the intensity of corresponding protein fingerprint peaks in normalized protein profiles with Biomarker Wizard analysis, some protein fingerprint peaks between HCC and hepatitis or cirrhosis were found to be significantly different ($P < 0.05$). The *P* value represented the power of each peak in discriminating HCC from hepatitis or cirrhosis. From these, some leading differential proteins between HCC and cirrhosis or chronic hepatitis B (average intensity ration > 2 or < 0.5) can be selected for future identification. Analysis of these leading differential proteins in subtraction difference mode, common differential proteins among three diseases, which showed potential specificity and clinical significance in dictator for HCC, can be determined successfully.

RESULTS

All protein spectra were normalized and aligned under the same parameters using Ciphergen SELDI Software 3.1.1 with Biomarker Wizard, and then auto-identified protein peaks were visually examined to minimize mismatched peaks between HCC and hepatitis or cirrhosis. Under the condition of signal-to-noise > 5 and minimum threshold for cluster > 20%, the sum of 128 serum protein peaks between 2 and 30 kDa were identified.

Comparison of serum protein spectra between HCC with HBV/cirrhosis and cirrhosis

Eighty-seven of 128 protein peaks were shown to be significantly different in intensity between HCC and cirrhosis after Biomarker Wizard software analysis ($P < 0.05$). Of the above differential proteins, forty-five differential proteins had over two-fold changes, which were defined as leading differential proteins, including 15 up-regulated proteins (m/z 8696, 13 769, 13969, 28060, 8772, 23 415, 22 857, 9354, 9730, 11 702, 9422, 9406, 2143, 9195, 5058) and 30 down-regulated proteins (m/z 2688, 2870, 3246, 3450, 3493, 3654, 4178, 4965, 2546, 4160, 3165, 2530, 2026, 2361, 3378, 3941, 5483, 2892, 3063, 3714, 2513, 2904, 2615, 2674, 2590, 3513, 3091, 5341, 3356, 8143) in HCC serum. However, the expression of eight up-regulated proteins (m/z 13 769, 13 969, 28 060, 9354, 9422, 9406, 2143, 9195, 5058) also increased in serum of HBV patients in this study, which suggested up-regulation of these eight common differential proteins might have reflected the pathological changes of hepatitis B, but were non-specific for HCC development.

Comparison of serum protein spectra between HCC with HBV/cirrhosis and chronic hepatitis B

Using Biomarker Wizard software analysis, nine of 52 differential proteins ($P < 0.05$), including two up-regulated proteins (m/z 3588, 2017) and seven down-regulated proteins (m/z 2870, 3654, 3941, 2688, 3450, 3165, 5483) in HCC serum, were found to be over twofold different in intensity between HCC and chronic hepatitis B. Compared with hepatitis patients, the expression of m/z 3588 and 2017 was also up-regulated in the serum of cirrhosis patients (Figure 1, Table 1). Therefore, these two common leading differential proteins may play a role in the progress of HBV-induced or cirrhosis-induced HCC, which merits further investigation. The other seven downregulated proteins in HCC serum were all detected to be downregulated in HCC serum as compared with serum protein spectra of cirrhosis (Figure 1, Table 1). This demonstrated that these common differential proteins possessed potential specificity and clinical significance in HCC diagnosis because they eliminate the influence of hepatitis and cirrhosis under subtraction difference analysis. However, expression of the two other leading differential proteins (m/z 3654, 3450) was higher in cirrhosis than in HCC and hepatitis, which indicated they were more specifically associated with cirrhosis.

Comparison of serum protein spectra between cirrhosis and chronic hepatitis B

Between cirrhosis and chronic hepatitis B, 28 of 79 significant differential proteins ($P < 0.05$) were found to have a greater than twofold change in intensity, including

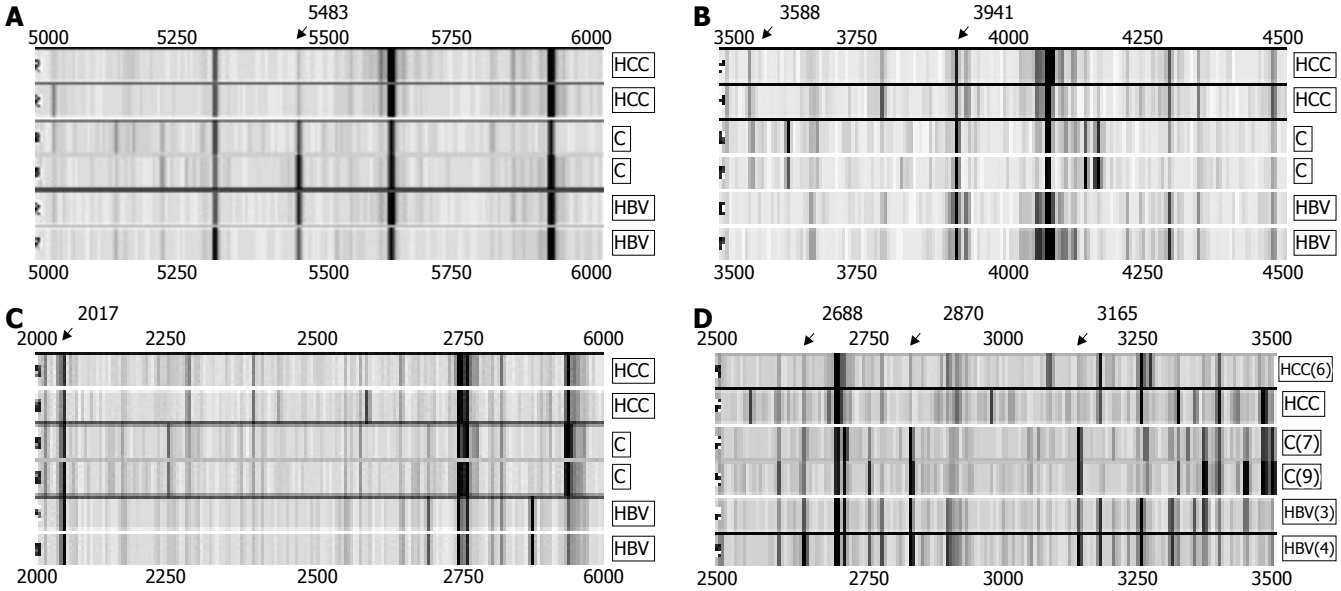


Figure 1 SELDI spectra of serum samples from patients with HCC with HBV/cirrhosis, cirrhosis and chronic hepatitis B. Arrows represent seven common leading differential proteins with low MW, including five common down-regulated proteins in HCC serum (m/z 2870, 3941, 2688, 3165, 5483) and two common up-regulated proteins (m/z 3588, 2017) in HCC and cirrhosis, using Ciphergen SELDI Software 3.1.1 with Biomarker Wizard and subtraction difference analysis. The intensity of each band in the gel view reflects the peak area in the original spectrum. HCC, HCC with HBV/cirrhosis; C, cirrhosis; HBV, hepatitis B virus.

17 up-regulated proteins (m/z 4178, 4965, 3493, 2892, 3476, 3246, 2017, 3714, 3279, 3450, 5003, 3588, 3513, 2513, 2904, 5810, 3654) and 11 down-regulated proteins (m/z 13769, 4075, 13969, 7772, 9291, 9422, 9354, 9406, 28060, 5058, 9195) in cirrhosis. Among 17 up-regulated differential proteins, expression of 11 (m/z 4178, 4965, 3493, 2892, 3246, 3714, 3450, 3513, 2513, 2904, 3654) also increased in cirrhosis, as compared to HCC, which indicated these proteins might have been specifically related to cirrhosis.

DISCUSSION

Several lines of evidence have shown that disease-related proteins or peptide fragments, produced by the tumor or tumor-host microenvironment, either at tumor onset or later, are readily released into the blood as it bathes or circulates through diseased tissue[13,18,19]. Therefore, blood can be used as a rich source of tumor biomarkers for the clinical diagnosis of many tumors. Over the years, many clinical blood tests based on serum markers have been developed for the detection of cancer[20-22]. For example, increased serum levels of CA125 (carbohydrate antigen 125), CA153 (carbohydrate antigen 153) and PSA (prostate specific antigen) are routinely used in clinics for detection of cancer in the ovary, breast and prostate, respectively. AFP, AFP-L3 and DCP are the most useful serum tumor markers for the diagnosis of HCC[23-25], and combination of these three tumor markers can improve the diagnostic accuracy, especially in differentiating HCC from non-malignant liver disease[12]. However, none of these biomarkers meets the goal of early diagnosis of HCC, or has satisfactory accuracy in detecting HCC and predicting prognosis when used alone. Recently, Wright[26] *et al* have summarized traditional and novel serum markers for HCC by review of the latest literature (2001-206). They predicted that in the near future a new generation of HCC serum markers, including complex proteomics features and tumor specific autoantibodies, will have the potential to replace its flawed leader, AFP, after strict validation in properly controlled clinical studies.

In the past, 2DE coupled with MS was the major technology in biomarker screening of serum proteomes. Following this classic screening pathway, 11 serum proteins with differential expression that is increased or decreased in response to HCC treatment have been identified. These proteins might be involved in liver carcinogenesis or become candidate biomarkers for the development of diagnostic and therapeutic tools[27]. In another recent study, two different screening strategies (2-DE combined with nano-HPLC-ESI-MS/MS or nano-HPLC-ESI-MS/MS) were simultaneously applied to discover more HCC-related proteins. This work means that scientists are beginning to address the shortcomings of 2DE in clinical proteomic studies of serum tumor marker screening[28]. 2DE technology is labor intensive, time-consuming, and requires a large amount of sample, therefore, it is not suitable for large-scale serum screening programs and clinical testing[29]. Furthermore, proteins with a low MW and abundance, and extreme pIs exceed the analytical capability of 2DE, although such proteins convey valuable information for disease assessment. As a result of its pre-fractionation, high resolution and powerful spectrum analysis software, application of SELDI-based systems can remedy some of the drawbacks of 2DE. They also have significant advantages over other proteomics technology for serum biomarkers screening.

For specific biomarkers discovery of HCC with HBV(+)/cirrhosis(+) in serum, two challenging aspects should be addressed (1) to eliminate the interference of most unspecific abundant proteins secreted by liver damage such as hepatitis, cirrhosis, etc., which were also 'reasonable'

differential proteins as compared with health controls (2) to reduce the complexity of serum proteome as more as possible so that most uncharacterized proteins with low abundance and low MW can be detected in serum. Serum proteins have a range of concentrations and are dominated by a handful of high-abundance proteins such as albumin, transferrin, haptoglobulin, immunoglobulins and lipoproteins[13]. Therefore, identification of low-abundance biomarkers is generally hampered by the presence of a few more abundant proteins. Pre-fractionation with simple chemical affinity beads or surface is an effective method to decrease the sample complexities. In this study, chip protein fractionation coupled to high resolution SELDI-TOF-MS not only reduced the complexity of serum proteomes prior to MS analysis, but also improved detection of uncharacterized proteins with low abundance and MW. On the other hand, subtraction difference analysis attenuated the influence of non-specific abundant proteins secreted by patients with hepatitis B or cirrhosis, and guaranteed that candidate tumor biomarkers screened were more specifically for HCC. Through mutually comparative analysis of serum SELDI spectra among three disease groups, the five common down-regulated proteins in HCC serum (m/z 2870, 3941, 2688, 3165, 5483) and another two common up-regulated proteins (m/z 3588, 2017) in HCC and cirrhosis serum were screened, and showed potential clinical significance for diagnosis of HCC (Figure 1, Table 1). Because most studies for HCC biomarker discovery mainly focused on the changes of its serum SELDI spectra in comparison with that of hepatitis B/C or cirrhosis alone, or between non-HCC and HCC patients[16,17,30], some identified differential proteins probably reflect the pathological alteration of hepatitis or cirrhosis only, but unspecific for HCC development[16,26]. Therefore, the above seven common differentially expressed proteins, screened in HCC serum, undoubtedly possessed an advantage in specificity for evaluating the pathological stage of HCC. More recently, applying combination technology of enrichment, SELDI and LC-MS/MS or SELDI, 2-DE as well as LC-MS/MS, some candidate biomarkers for HCV-related HCC such as complement C3a[16], κ and λ immunoglobulin light chains[30], *etc*. were identified in succession. These identification studies will offer a valuable reference to characterize these seven common differential proteins for HCC diagnosis in our proceeding study.

In summary, common differential candidate proteins screened here among three disease groups under SELDI system and subtract difference analysis not only show potential clinical significance in HCC indicator, but also provide a novel option to discover specific biomarkers in serum of HCC.

COMMENTS

Background

Although some serum tumor markers including enzymes (GGT, AFU, DCP), and oncofetal and glycoprotein antigens (AFP, AFP-L3, GPC3) have been widely used in the detection of HCC, the diagnostic accuracy for HCC is still unsatisfactory, especially for early and differential diagnosis. There is an urgent need to discover new specific HCC-associated proteins, using novel technology and screening strategies. For biomarkers for diagnosis of HCC with HBV/cirrhosis, two challenging questions remain: (1) how to eliminate the interference of most non-specific abundant secreted proteins from hepatitis and cirrhosis; and (2) how to reduce the complexity of serum proteome, which hinders the discovery of specific HCC-associated serological proteins.

Research frontiers

A promising high throughput proteomics approach, on-chip protein fractionation coupled to SELDI-TOF-MS, provides a novel pathway for rapid screening and identification of specific tumor markers with low MW in the proteomes of body fluids.

Innovations and breakthroughs

Applying SELDI and subtraction difference analysis, we successfully discovered seven common differential candidate proteins with potential clinical significance for HCC indicator. This screening strategy provides a novel method for discovering specific biomarkers with low MW and low abundance in the serum of patients with HCC.

Applications

These seven common differential proteins with potential clinical significance for HCC diagnosis merit being further identified and validated in large numbers of HCC samples.

Terminology

SELDI-TOF-MS detects proteins affinity-bound to a protein chip array. This system is a novel, sensitive, rapid detection system for analyzing complex mixtures of proteins and peptides.

Peer review

This study investigated serum HCC-associated proteins with low MW and low abundance in patients with HCC with HBV/cirrhosis by SELDI-based serum protein spectrum analysis. Seven common differential candidate proteins with potential clinical significance in diagnosis of HCC were discovered by subtraction difference analysis. These preliminary results and subtraction screening strategy will provide a useful reference for the discovery of biomarkers for HCC.

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RAPID COMMUNICATION

Efficacies of β-L-D4A against Hepatitis B virus in 2.2.15 cells

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Abstract

AIM: To investigate the antiviral effect of beta-L-enantiomer of 2′,3′-didehydro-2′,3′-dideoxyadenosine (β-L-D4A) on 2.2.15 cells transfected with the hepatitis B virus (HBV) genome.

METHODS: Lamivudine (3TC) as a positive control. Then, HBV DNA in treated 2.2.15 cells and the Hepatitis B surface antigen (HBsAg) in the culture supernatants were detected to determine the inhibitory effect of β-L-D4A. At the same time, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to detect the survival ratio of 2.2.15 cells.

RESULTS: β-L-D4A has a dose-dependent inhibitory effect on HBV DNA replication; this effect was apparent when the concentration was above 1 mol/L. When β-L-D4A was at the highest concentration, 100 mol/L, the HBsAg inhibition ratio was above 50%. The Therapeutic index (TI) of β-L-D4A was above 2.1.

CONCLUSION: β-L-D4A has a dose-dependent inhibitory effect on the replication of HBV DNA and the secretion of HBsAg at low toxicity.

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Key words: 2′,3′-didehydro-2′,3′-dideoxyadenosine; 2.2.15 cells; Hepatitis B virus DNA; Hepatitis B surface antigen

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INTRODUCTION

Hepatitis B virus (HBV) is a partially double-stranded DNA virus, which is able to induce acute or chronic infection in humans[1]. Chronic HBV infection can last a lifetime, causing persistent hepatitis that frequently progresses to more-severe liver diseases[2], including cirrhosis and hepatocellular carcinoma[3-5]. Chronically infected patients also serve as sources of HBV transmission.

Traditionally approved therapies against HBV include treatment with alpha interferon and lamivudine (3TC). However, the efficacy of alpha interferon is limited by undesirable side effects, low sustained response rate and high cost[6-7]. While lamivudine (3TC) is well-tolerated and less costly, resistance emerges in approximately 20% of patients receiving monotherapy per year[8].

Adefovir-dipivoxil prodrug (ADV) and entecavir (ETV) are recently approved anti-HBV therapeutics. While these two agents produce multilog suppression of serum HBV DNA, they cause only modest rates of HBV surface antigen seroconversion and, thus, require long-term administration to control disease in most patients[9]. ADV retains clinical efficacy against lamivudine-resistant mutants[10], but selects the mutations rtN236T and rtA181V[11]. In addition, treatment with ADV is reported to be associated with dose-limiting nephrotoxicity[12]. Entecavir shows reduced susceptibility to lamivudine-resistant mutants *in vitro* and clinically, and an accelerated development of entecavir resistance mutations (rtI169T, rtM250V, rtS202I and rtT184S/G) in lamivudine-resistant patients[12,13]. Therefore, more effective and safer therapies are needed for the treatment of chronic HBV infections.

β-L-D4A is an L-enantiomer of the natural nucleoside deoxyadenosine. The structure of β-L-D4A is shown in Figure 1. In this report, we detected the efficacy of β-L-D4A against HBV replication *in vitro*.

MATERIALS AND METHODS

Materials

2.2.15 cells and 3TC were kindly provided by Professor Yung-Chi Cheng (Yale University) and plasmid P3.6 was offered by Professor Wang Yuan (Shanghai Institute of

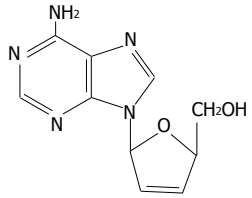


Figure 1 Chemical structure of β-L-D4A.

Biochemistry, Chinese Academy of Sciences). β-L-D4A was synthesized by the Department of Pharmacology of Wuhan University. G418 and MTT were purchased from Sigma (USA) and DMEM was from Gibco (USA). Fetal bovine serum was purchased from Hyclone (USA), random primers were purchased from Takara (Japan) and DIG-labeled dUTP was purchased from Roche (Germany). An EIA Kit for the detection of HBsAg was purchased from Kehua (Shanghai). HBV DNA fluorescent quantified PCR (FQ-PCR) Kit was purchased from Biotronics (USA).

Methods

Cell culture: 2.2.15 cells were maintained in DMEM (supplemented with 10% fetal bovine serum, 200 g/mL G418, 2 mmol/L glutamine, 50 U of penicillin per milliliter and 50 μg of streptomycin per milliliter) at 37℃ and 5% CO_2. The medium was changed every three days.

Intracellular HBV DNA analysis: 2.2.15 cells were digested by parenzyme, seeded at a density of 1×10^5 cells per well on 24-well plates and maintained in 1.5 mL DMEM at 37℃ and 5% CO_2 for 48 h. Then, 2.2.15 cells were treated with freshly prepared medium containing β-L-D4A or 3TC. The following concentrations were used: 0.0001, 0.001, 0.01, 0.1, 1, 10, 100 mol/L. Negative control cells were treated with DMEM only. Media were changed every three days; on day 8, both the supernatants and cells were collected and stored at -20℃.

Then, the cells were lysed at room temperature with 1 mL of lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 0.2% Nonidet P-40]. Removal of cellular debris was accomplished by a 5-min microcentrifuge spin at 16 000 r/min. Lysates were then incubated with 20% PEG 8000 and 1 mol/L NaCl at room temperature for 2 h, followed by a 1-min microcentrifuge spin at 16 000 r/min. The resulting pellets containing HBV core particles were resuspended in solution added with proteinase K [10 mmol/L Tris (pH 7.6), 10 mmol/L EDTA, 0.25% SDS, 1 mg/mL proteinase K] at 56℃ for 2 h, followed by extraction with phenol-chloroform and ethanol precipitation. Nucleic acids were dissolved in ddH_2O, electrophoresed through 1% agarose, transferred to a positively charged nylon membrane and hybridized to a probe, which was prepared from a full-length HBV DNA genome template excised from plasmid P3.6 and DIG-labeled using a random primer. Then, the membrane was incubated with anti-DIG and developed with NBP/ BCIP.

The concentration of extracted HBV DNA was also determined by real-time fluorescent quantitative Polymerase Chain Reaction(FQ-PCR). The inhibition ratio was calculated according to the following formula: inhibition ratio = (HBV DNA concentration of negative control - HBV DNA concentration of experimental group)/HBV DNA concentration of negative control × 100%. Then, the 50% inhibition concentration (IC_{50}) was calculated according to the Reed-Muench method[14].

Detection of HBsAg: 2.2.15 cells were treated with antiviral compounds and lysed as described above. The collected supernatants were detected using an EIA Kit for the detection of HBsAg according to the manufacturer's instructions, and A_{450} was observed. Inhibition ratio to HBsAg (%) = (A_{450} of negative control -A_{450} of experimental group) / (A_{450} of negative control -A_{450} of blank control) × 100%.

Analysis of cellular toxicity: MTT assays were used to detect the survival rates of 2.2.15 cells[15]. Briefly, 2.2.15 cells were seeded at a density of 1×10^5 cells per well on 24-well plates and treated with media containing β-L-D4A or 3TC of different concentrations. The following concentrations were used: 1000, 500, 250, 125, 62.5 and 31.25 mol/L. Three days later, the supernatants were discarded and 2 mL of MTT (1 mg/mL) was added per well. After incubation at 37℃ and 5% CO_2 for 4 h, the supernatants were discarded and 2 mL of acidified isopropanol (isopropanol:HCl = 500:1.68) was added per well. The survival ratio of 2.2.15 cells (%) = (A_{570} of experimental group/A_{570} of negative control) × 100%. Then, the 50% toxicity dose (TD_{50}) was calculated according to the Reed-Muench method.

Evaluation of treatment: Therapeutic index (TI) = TD_{50}/IC_{50}. If the TI is above 2.1, the compound is high in efficacy and low in cellular toxicity. If TI is between 2 and 1, the compound is both considerably effective and toxic. If TI is below 1, the compound is low in efficacy, but high in cellular toxicity.

Statistics analysis

All statistics were analyzed using SPSS10.0, and differences were considered significant when the P value was < 0.05.

RESULTS

β-L-D4A inhibits the replication of HBV DNA

To investigate the potential of β-L-D4A to inhibit HBV viral replication, HBV DNA was isolated from treated 2.2.15 cells and detected by Southern hybridization. Figure 2 shows that the production of HBV DNA lessened following treatment of cells with β-L-D4A; this effect became more notable as the dose of β-L-D4A increased. A similar phenomenon was observed in cells treated with 3TC.

The concentration of HBV DNA in 2.2.15 cells was determined by real-time fluorescent quantitative PCR. As shown in Figure 3, β-L-D4A had an apparent inhibitory effect on the concentration of HBV DNA ($P > 0.05$ when compared to that of 3TC). Calculated according to the Reed-Muench method, the IC_{50} values of β-L-D4A and 3TC were 0.61 mol/L and 0.30 mol/L, respectively.

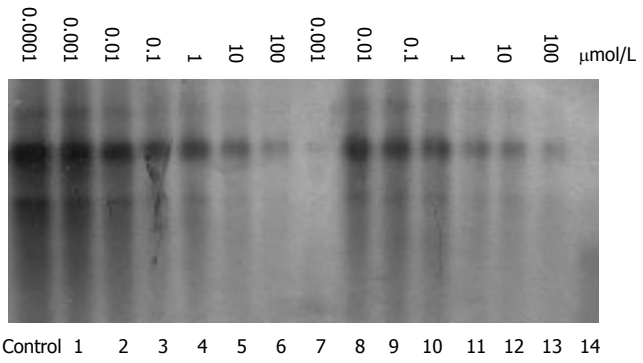


Figure 2 Southern analysis of the HBV DNA extracted from 2.2.15 cells. Lane control: HBV DNA extracted from untreated 2.2.15 cells; Lane 1-7: HBV DNA extracted from 2.2.15 cells treated with β-L-D4A; Lane 8-14: HBV DNA extracted from 2.2.15 cells treated with 3TC.

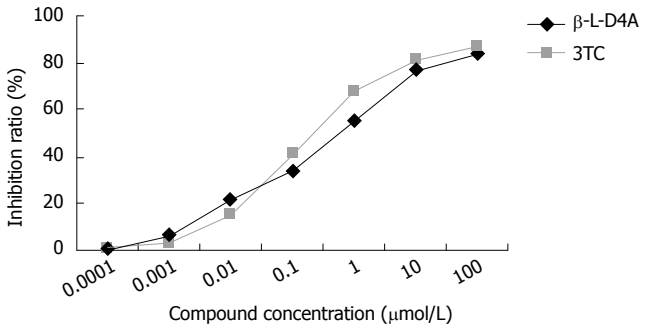


Figure 3 The inhibition ratio to HBV DNA.

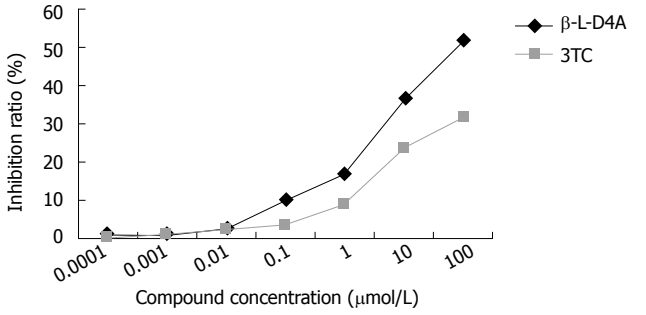


Figure 4 The inhibition ratio to HBsAg.

β-L-D4A inhibits the secretion of HBsAg

Following treatment of 2.2.15 cells with antiviral compounds for eight days, HBsAg was detected in the medium. As shown in Figure 4, when the concentration of β-L-D4A was 100 mol/L, the HBsAg inhibition ratio was above 50%. However, the HBsAg inhibition ratio with 3TC was less than 40% even at the highest concentration.

Cellular toxicity of β-L-D4A

The cellular toxicity of both compounds was not evident at drug concentrations below 125 mol/L. The survival ratios were less than 50% when the drug concentrations were as high as 1000 mol/L, with both compounds. Calculated according to the Reed-Muench method, the TD_{50} values of β-L-D4A and 3TC were 417 mol/L and 243 mol/L, respectively.

Table 1 TIs of β-L-D4A and 3TC

Group	IC_{50} (μmol/L)	TD_{50} (μmol/L)	TI
β-L-D4A	0.61	417	683
3TC	0.30	243	810

TI: Therapeutic index.

Evaluation of treatment

Table 1 shows the TIs for both β-L-D4A and 3TC were above 2.1. This means that these two compounds are high in efficacy and low in cellular toxicity.

DISCUSSION

HBV infection is one of the most prevalent viral diseases in the world and more than 350 million people are chronically infected[16,17], with about 1 million deaths annually[18]. In order to screen anti-HBV compounds, researchers often purify HBV DNA polymerase. However, the purification procedure is very complicated and the quantity of polymerase is not satisfying[19,20]. 2.2.15 cells can stably replicate HBV DNA and express HBsAg; thus, they can be used as an ideal cell model to screen the *in vitro* antiviral efficacy of medicines[21,22].

In this study, we found that β-L-D4A has a dose-dependent inhibitory effect on HBV DNA replication that is apparent when the concentration is above 1 mol/L. However, the inhibition of the secretion of HBsAg was not as pronounced as that of HBV DNA replication. When β-L-D4A was at the highest concentration, 100 mol/L, the HBsAg inhibition ratio was slightly above 50%. The TI of β-L-D4A shows that it is high in efficacy and low in toxicity, which indicates that β-L-D4A is a promising anti-HBV compound.

β-L-D4A is a new type of L-nucleoside for which the natural counterpart is deoxyadenosine. Although there have been reports about L-nucleosides since 1960s, it was only after about 30 years that the merits of L-nucleoside became widely recognized[23-24]. Previous studies revealed the bioactivities and securities of L-nucleoside analogues were superior to their counterpart enantiomers[25-27]. β-L 3/(2R, 5S)-1,3-oxathiolane-cytidine (lamivudine, 3TC), a typical L-nucleoside, was reported to be about 50 times more effective than its D-enantiomer[28-30].

The mechanism of action of β-L-D4A is speculated to be inhibition of HBV DNA polymerase and termination of DNA replication. In order to confirm this speculation, further study is necessary. It also remains to be seen whether β-L-D4A is effective against lamivudine-resistant HBV mutants and whether it will lead to HBV mutants resistant to β-L-D4A. Besides, much work remains to be done to gain a better understanding of the metabolism, toxicology and pharmacokinetic parameters of this compound. Our studies to date suggest β-L-D4A deserves further development as a potential treatment for chronic HBV infection.

ACKNOWLEDGMENTS

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2.2.15 cells and 3TC. We also thank Professor Wang Yuan for the plasmid P3.6.

COMMENTS

Background

Hepatitis B virus (HBV) is one of the causative agents of acute or chronic hepatitis in humans, and there are an estimated 350 million persistently HBV-infected carriers worldwide. Such prevalence of HBV demands the discovery of new effective therapeutics for the treatment of acute and chronic infections.

Research frontiers

There have been articles studying the antiviral effects of Entecavir and Adefovir, which are analogs of deoxyguanosine and deoxyadenosine, respectively. While these two agents produce pronounced suppression of serum HBV DNA, they cause only modest rates of HBV surface antigen seroconversion and even induce resistant mutants. Thus, there is an urgent need to develop a new potent anti-HBV drug.

Innovations and breakthroughs

In this study, we find that β-L-D4A has a dose-dependent inhibitory effect on the replication of HBV DNA and the secretion of HBsAg at low toxicity.

Applications

The results of this study confirm the *in vitro* antiviral effect of β-L-D4A, which indicates it is a promising new drug against HBV infection.

Terminology

β-L-D4A is an L-enantiomer of its natural nucleoside-deoxyadenosine.

Peer review

This study aimed to investigate the antiviral effect of β-L-D4A on 2.2.15 cell line by comparing with 3TC. They found β-L-D4A has concentration-relative inhibitory effect to HBV DNA replication and the therapeutic index of β-L-D4A was above 2.1. It's an interesting subject.

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RAPID COMMUNICATION

Effects of antiviral agents and HBV genotypes on intrahepatic covalently closed circular DNA in HBeAg-positive chronic hepatitis B patients

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Abstract

AIM: To evaluate the effects of antiviral agents and HBV genotypes on intrahepatic covalently closed circular DNA (ccc DNA) in HBeAg-positive chronic hepatitis B patients.

METHODS: Seventy-one patients received lamivudine (n = 35), or sequential therapy with lamivudine- interferon alpha 2b (IFN-α 2b, n = 24) for 48 wk, or IFN-α 2b (n = 12) for 24 wk. All subjects were followed up for 24 wk. Intrahepatic ccc DNA was measured quantitatively by PCR. HBV genotypes were analyzed by PCR-RFLP.

RESULTS: Sequential lamivudine- INF-α therapy, lamivudine and INF-α monotherapy reduced ccc DNA of 1.7 log, 1.4 log and 0.8 log, respectively (P < 0.05). Seventeen out of the 71 patients developed HBeAg seroconversion, the reduction of ccc DNA in the HBeAg seroconversion patients was more significant than that in the HBeAg positive patients (3.0 log *vs* 1.6 log, P = 0.0407). Twenty-four weeks after antiviral therapy withdrawal, 16 patients had a sustained virological response, the baseline intrahepatic ccc DNA in the patients with a sustained virological response was significantly lower than that in the patients with virological rebound (4.6 log *vs* 5.4 log, P = 0.0472). HBV genotype C accounted for 85.9% (n = 61), and genotype B for 14.1% (n = 10), respectively, in the 71 patients. There was no significant difference in the change of ccc DNA level between HBV genotypes C and B (2.1 log *vs* 1.9 log).

CONCLUSION: Forty-eight week sequential lamivudine-INF-α therapy and lamivudine monotherapy reduce ccc DNA more significantly than 24-wk INF-α monotherapy. Low baseline intrahepatic ccc DNA level may predict the long-term efficacy of antiviral treatment. HBV genotypes C and B have no obvious influence on ccc DNA load.

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Key words: Covalently closed circular DNA; Hepatitis B virus; Sequential therapy; Lamivudine; Interferon

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INTRODUCTION

Hepatitis B virus (HBV) is one of the major causes for liver disease, more than 350 million people are infected with HBV, and eventually 15% to 25% of these individuals will progress to liver cirrhosis and hepatocellular carcinoma[1]. Interferon-alpha (IFN-α)[2-4] and nucleoside analogue agents[5-8] are the currently available antiviral drugs for the treatment of patients with chronic hepatitis B. Long-term anti-viral treatment can decrease the occurrence rate of hepatocellular carcinoma in patients with chronic hepatitis B, thus improving their survival. Nonetheless, after withdrawal of treatment, relapse occurs in the great majority of patients. Furthermore, relapse may lead to severe exacerbation of the disease, causing hepatic failure and even death. The main reason for the rebound of HBV DNA to its pretreatment level after antiviral

treatment withdrawal is that these agents have a profound effect on relaxed circular DNA (rc DNA) and almost no effect on covalently closed circular DNA (ccc DNA)[9-11]. ccc DNA provides the template for the transcription of all viral genes. After hepatocytes are infected with HBV, covalently closed circular DNA is formed through DNA repair of the relaxed circular replicative HBV DNA inside the nuclei of hepatocytes[12]. DNA-containing nucleocapsids can either recycle back to the nuclei to amplify and maintain the pool of ccc DNA or become enveloped and are secreted into the blood[13]. Since ccc DNA is required for the maintenance of HBV infection, it must be eliminated for viral clearance to present. Currently, ccc DNA in human peripheral blood mononuclear cells and liver biopsies can be quantitated [14-16]. Thus, it is possible to measure the intrahepatic ccc DNA level for monitoring antiviral treatment.

The aim of the present study was to analyze the effects of sequential lamivudine-INF-α therapy, lamivudine monotherapy and INF-α monotherapy on intrahepatic ccc DNA. The relationship between intrahepatic ccc DNA and HBV genotypes was also observed.

MATERIALS AND METHODS

Subjects

Between March 2003 and March 2005, seventy-one patients with HBeAg -positive chronic hepatitis B were recruited, including 59 males and 12 females aged 19-47 (mean 32 ± 9) years. Informed consent was obtained from all involved patients. The inclusion criteria for the patients were as follows: positive for HBsAg and HBeAg, serum HBV DNA $\geqslant 1 \times 10^5$ copies/L, serum alanine aminotransferase (ALT) levels at least two-fold higher than the normal range (normal range = 0-667 nkat/L) for more than 6 mo. Exclusion criteria were as follows: patients with alcoholism, pregnancy, cirrhosis, chronic renal failure, concurrent autoimmune disease, severe neurological disorders, human immunodeficiency virus (HIV) infection and viral hepatitis A, C, delta or E. Patients treated with interferon or other antiviral therapies for 6 mo prior to enrollment in this study were also excluded. Patients were randomized (at the ratio 3:2:1) to receive 48 wk of 100 mg lamivudine once daily (n = 35), or lamivudine from the first to the 8th mo and IFN-α from the 7th to the 12th mo (n = 24), or 24 wk of 5 million units of interferon-alpha (IFN-α) three times per week (n = 12). All subjects were followed up for 24 wk after antiviral therapy. Presence of serum HBV DNA levels $\leqslant 1 \times 10^3$ and normalization of serum ALT levels were assessed as treatment response.

Measurement of HBV markers and genotypes and biochemical tests

Blood samples were obtained before, immediately after treatment and at wk 24 of follow-up. Liver biochemistry and HBV marker test (using commercial assays, Abbott Laboratories, USA) were performed on these samples.

Liver biopsy specimens were collected by needle liver biopsies (0.5-1.5 cm) before and immediately after treatment. The tissue was washed several times in cold phosphate-buffered saline (PBS) and stored at -70℃. Total DNA was extracted from liver tissue with the Qiaamp DNA tissue Mini DNA kit (Qiagen, Germany). Serum and intrahepatic HBV DNA were measured quantitatively by real-time polymerase chain reaction (PCR) (Model 5700, ABI Company, USA) with a lower limit of detection of 1×10^3 HBV DNA copies/mL and 1×10^3 copies/μg total DNA, respectively. HBV genotypes were determined by PCR restriction fragment length polymorphism (PCR-RFLP) analysis as previously described[17].

PCR amplification for CCC DNA

The primers were synthesized for amplifying ccc DNA according to the published consensus sequence. Sequences of the primers and the probe are as follows: 5'-ATACGGGTCAATGTCCATGC-3' (nt 1551, sense), 5'-CCGTCTGTGCCTTCTCATCT-3' (nt 1991, antisense), and 5'-CCAAAGCCACCCAAGGCACA-3' (nt 1880, probe).

PCR was performed at 94℃ for 2 min, followed by 45 cycles at 94℃ for 20 s, at 60℃ for 1 min.

Histology measurement

Histologic inflammation was scored with a modified Knodell scoring system[18]. Histologic response was defined as a decrease by at least two points in the Knodell scoring system.

Statistical analysis

Data were analyzed with the Statistical Program for Social Sciences (SPSS 13.0 for Windows). Categorical variables were tested using chi-square test or Fisher's exact test. Normally distributed variables were tested using t-test or ANOVA, whereas continuous variables with skewed distributions were tested using the Kruskal Wallis test. Correlation between two variables was tested using Pearson's correlation analysis after logarithmic transformation of data with skewed distributions. $P < 0.05$ was considered statistically significant.

RESULTS

There was no significant difference in all the parameters among the three groups before treatment. The antiviral outcome of 48-wk sequential lamivudine - INF-α therapy was similar to that of 48-wk lamivudine monotherapy, but slightly better than that of 24-wk INF-α monotherapy. The reduction in serum HBV DNA, intrahepatic HBV DNA and ccc DNA after sequential lamivudine - INF-α therapy and lamivudine monotherapy was greater than that after INF-α monotherapy ($P < 0.05$) (Table 1).

Data on histology, biochemistry and virology in HBeAg seroconversion patients and HBeAg positive patients

After antiviral treatment, the means of Knodell score, serum HBV DNA levels, intrahepatic HBV DNA levels, intrahepatic ccc DNA and serum ALT levels in all the 71 patients were significantly decreased ($P < 0.05$). Of the 71 patients, 17 developed HBeAg seroconversion. There was no significant difference in the parameters mentioned

Table 1 Demographic, histological, biochemical, virological data obtained from three antiviral therapies (mean ± SD)

Parameter	Sequential lamivudine -INF-α therapy	Lamivudine monotherapy	Interferon monotherapy
Number of patients	24	35	12
Sex ratio (male/female)	21/3	29/6	9/3
Mean age (y)	33 ± 6	32 ± 5	31 ± 8
Mean Knodell score			
pretreatment	8.9 ± 3.6	8.2 ± 4.5	7.1 ± 4.0
post-treatment	6.6 ± 4.0[a]	5.6 ± 3.2[a]	5.0 ± 2.8
Mean ALT level (nkat/L)			
Pretreatment	3423 ± 2388	4390 ± 2454	3006 ± 1720
Post-treatment	668 ± 400[a]	667 ± 384[a]	1686 ± 1286[a]
Serum HBV DNA (log10)			
Pretreatment	7.5 ± 1.3	7.7 ± 0.8	7.8 ± 1.1
Post-treatment	3.8 ± 1.0[a]	3.7 ± 1.2[a]	5.0 ± 1.5[a,c]
Intrahepatic HBV DNA (log10)			
Pretreatment	5.8 ± 1.2	6.2 ± 0.9	6.3 ± 0.9
Post-treatment	4.7 ± 1.1[a]	4.6 ± 1.5[a]	5.6 ± 1.5[a,c]
Intrahepatic ccc DNA (log10)			
Pretreatment	5.1 ± 1.0	5.2 ± 1.2	5.8 ± 1.2
Post-treatment	3.4 ± 1.3[a]	3.8 ± 1.1[a]	5.0 ± 1.5[c]
HBeAg seroconversion rate at the treatment completion	8/21 (38.1%)	6/32 (18.6%)	3/12 (25%)
Sustained virological response rate at 24 wk off treatment	4/16 (25%)	13/31 (41.9%)	1/1 (100%)

[a]$P < 0.05$ *vs* post-treatment, [c]$P < 0.001$ *vs* sequential lamivudine - INF-α therapy and lamivudine monotherapy. HBeAg seroconversion rate at the end of treatment: some post-treatment data were lost, ALT: Alanine aminotransferase, ccc DNA: Covalently closed circular DNA.

Table 2 Histological, biochemical and virological data on different therapy responses (mean ± SD)

Parameter	HBeAg positive	HBeAg serocon -version	Sustained virological response	Virological flares
Number of patients	48	17	18	30
Mean Knodell score				
Pretreatment	8.4 ± 4.1	7.1 ± 4.2	8.0 ± 4.0	8.4 ± 3.9
Post-treatment	6.0 ± 3.6	5.1 ± 3.1[a]	6.1 ± 3.0	5.2 ± 2.7[a]
Mean ALT level (nkat/L)				
Pretreatment	3206 ± 2338	3841 ± 2371	3340 ± 2638	4576 ± 2505
Post-treatment	1018 ± 985	618 ± 384[a]	517 ± 300	601 ± 400
24 wk off- treatment			851 ± 200	380 ± 183[e]
Serum HBV DNA (log10)				
Pretreatment	7.9 ± 0.9	7.5 ± 1.0	7.3 ± 1.3	7.6 ± 0.8
Post-treatment	4.4 ± 1.4[a]	3.0 ± 0.2[a,c]	3.1 ± 0.3[a]	3.3 ± 0.4[a]
24 wk off-treatment			3.2 ± 0.3	6.1 ± 0.8[a,c]
Intrahepatic HBV DNA (log10)				
Pretreatment	6.3 ± 0.8	5.6 ± 1.2	5.9 ± 0.8	6.0 ± 1.1
Post-treatment	5.1 ± 1.5	4.1 ± 0.8[a]	4.5 ± 1.6	4.3 ± 1.0[a]
Intrahepatic ccc DNA (log10)				
Pretreatment	5.5 ± 1.1	5.8 ± 1.0	4.6 ± 1.0	5.4 ± 0.8[e]
Post-treatment	3.9 ± 1.5	2.8 ± 1.3[a,c]	4.0 ± 0.9	3.8 ± 0.8[a]

[a]$P < 0.05$ *vs* post-treatment, [c]$P < 0.05$ *vs* HBeAg positive, [e]$P < 0.05$ *vs* sustained virological response. ALT: Alanine aminotransferase; ccc DNA: Covalently closed circular DNA.

above between the HBeAg seroconversion patients and HBeAg positive patients before treatment, except for the baseline intrahepatic HBV DNA levels (5.6 log *vs* 6.3 log, $P = 0.02$). The HBeAg seroconversion group had greater improvements than the HBeAg positive group. The mean of intrahepatic HBV DNA decreased to (4.1 ± 0.8) log10 in the seroconversion group ($P = 0.0124$) and to (5.1 ± 1.5) log10 ($P = 0.0872$) in the HBeAg positive group. The mean of intrahepatic ccc DNA decreased from (5.8 ± 0.6) log10 to (2.8 ± 1.3) log10 in the HBeAg seroconversion group, and from (5.5 ± 1.1) log10 to (3.9 ± 1.1) log10 in the HBeAg positive group ($P = 0.0407$, Table 2).

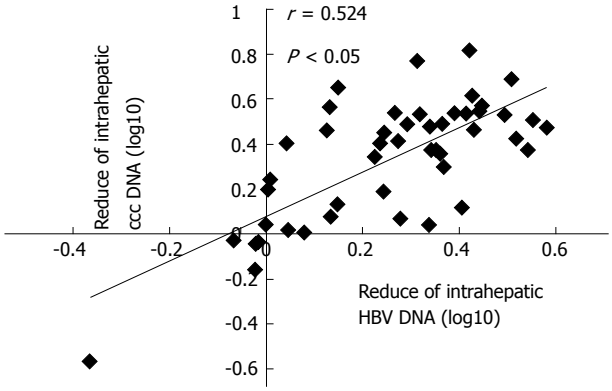


Figure 1 Correlation between the reduction in intrahepatic ccc DNA and a similar reduction in intrahepatic HBV DNA.

Data on histology, biochemistry and virology in patients with different long-term responses

In the 24-wk follow-up period, of the 48 patients with virological response at the end of therapy, 18 achieved a sustained virological response, and 30 presented virological rebound. The means of intrahepatic and serum HBV DNA loads, histology and serum ALT level in the patients with a sustained virological response were similar to those in the patients with virological rebound before and after treatment. The baseline intrahepatic ccc DNA level in the patients with a sustained virological response was, however, significantly lower than that in the patients with virological rebound ($P = 0.0472$), but no significant difference was found at the end of treatment between the two groups (Table 2).

The mean of HBV ccc DNA in the 71 patients before and after treatment was (6.83 ± 0.37) log and (5.72 ± 0.23) log, respectively ($P > 0.05$). The change in intrahepatic ccc DNA had a positive correlation with a similar reduction in intrahepatic HBV DNA ($r = 0.525$, $P < 0.05$, Figure 1). The ccc DNA level was correlated with the serum HBeAg titer at the end of treatment ($r = 0.432$, $P = 0.002$, Figure 2).

Intrahepatic ccc DNA level and HBV genotype

HBV genotype C accounted for 85.9% ($n = 61$), and genotype B for 14.1% ($n = 10$) in the 71 patients. The mean of intrahepatic HBV DNA loads was (6.1 ± 0.9) log10 and (4.9 ± 1.4) log10, respectively ($P < 0.05$), and that of intrahepatic ccc DNA loads was (5.6 ± 1.1) log10 and (3.5 ± 1.5) log10, respectively, in the genotype C patients before and after treatment ($P < 0.05$). The baseline and post-treatment intrahepatic HBV DNA level was (5.6 ± 1.5) log10 and (4.7 ± 1.2) log10, respectively

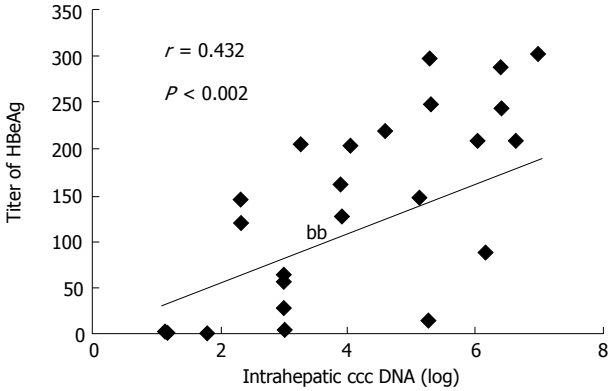


Figure 2 Relationship between intrahepatic ccc DNA level and HBeAg titer at the end of treatment.

($P > 0.05$), and that of intrahepatic ccc DNA loads was (5.8 ± 0.6) log10 and (3.9 ± 1.9) log10, respectively, in the genotype B patients ($P = 0.064$). No statistically significant difference was found in serum intrahepatic HBV DNA loads and ALT levels between the two groups after treatment. There was no significant difference in serum HBV DNA and alanine aminotransferase levels at 24 wk after treatment withdrawal, the sustained virological response rate and ALT normalization rate were also similar (Table 3).

Table 3 Histological, biochemical and virological data on HBV genotypes C and B (mean ± SD)

Parameter	HBV Genotype C	HBV Genotype B
Number of patients	61	10
Sex ratio (male/female)	50/11	9/1
Number of Sequential therapy	20 (32.8%)	4 (40%)
Number of Lamivudine therapy	31 (50.8%)	4 (40%)
Number of Interferon therapy	10 (16.4%)	2 (20%)
Mean Knodell score		
Pretreatment	8.1 ± 4.1	5.7 ± 3.2
Post-treatment	6.0 ± 3.4[a]	5.4 ± 3.1
Mean ALT level (nkat/L)		
Pretreatment	3390 ± 2438	2438 ± 2296
Post-treatment	901 ± 918	551 ± 233
24 wk off-treatment	1503 ± 1336	1987 ± 1720
Serum HBV DNA (log10)		
Pretreatment	7.7 ± 1.2	7.6 ± 0.8
Post-treatment	4.05 ± 1.3[a]	4.9 ± 1.2[a]
24 wk off-treatment	5.1 ± 1.6	5.3 ± 1.3
Intrahepatic HBV DNA (log10)		
Pretreatment	6.1 ± 0.9	5.6 ± 1.5
Post-treatment	4.9 ± 1.4[a]	4.7 ± 1.2[a]
Intrahepatic ccc DNA (log10)		
Pretreatment	5.6 ± 1.1	5.8 ± 0.5
Post-treatment	3.5 ± 1.2[a]	3.9 ± 1.9
Sustained virological response rate at		
24 wk off-treatment	29.4% (15/51)	30% (3/10)
ALT normalization rate at		
24 wk off-treatment	39.3% (24/51)	30% (3/10)

[a]$P < 0.05$ *vs* post-treatment. ALT: Alanine aminotransferase; ccc DNA: Covalently closed circular DNA.

DISCUSSION

It was reported that lamivudine reduces serum ccc DNA levels by magnitude of 2 log[19]. Forty-eight weeks after adefovir dipivoxil therapy resulted in a significant decrease of 0.8 log in ccc DNA copies/cell and changes in ccc DNA are correlated with a similar reduction in serum HBsAg titer[20]. A clinical study from Hong Kong reported that 48 wk treatment with entecavir ($n = 21$) and lamivudine ($n = 19$) can reduce serum viral load, intrahepatic total HBV DNA, and ccc DNA of about 4.8 logs, 2 logs, and 1 log, respectively[21]. In our study, 48 wk after sequential lamivudine - INF-α therapy ($n = 24$) and lamivudine monotherapy ($n = 35$), and 24 wk after INF-α monotherapy ($n = 12$) reduced serum HBV DNA of about 3.7 logs, 4 logs, and 2.8 log, intrahepatic total HBV DNA of about 1.1 logs, 1.6 logs and 0.7 log, ccc DNA of about 1.7 logs, 1.4 logs and 0.8 log, respectively ($P < 0.05$), indicating that either sequential lamivudine - INF-α therapy or lamivudine therapy significantly reduces HBV DNA and ccc DNA than INF-α monotherapy, and the relatively short duration of INF-α therapy may be responsible for such a difference.

Since serum HBV DNA and alanine aminotransferase levels often flare up after antiviral therapy even if the patients develop HBeAg seroconversion, it is important to determine what the parameters help assess the success of treatment and decide how long the therapy should be. The present data indicate that 17 out of the 71 patients developed HBeAg seroconversion after antiviral therapy, the change of ccc DNA in the HBeAg seroconversion patients was more significant than that in the HBeAg positive patients (3.0 log *vs* 1.6 log, $P = 0.0407$), suggesting that reduction in ccc DNA is crucial for chronic hepatitis patients to achieve better antiviral efficacy. Twenty-four weeks after antiviral therapy withdrawal, 18 patients achieved a sustained virological response. The baseline and post-treatment intrahepatic HBV DNA and serum HBV DNA levels in these patients were similar to those in the patients with virological rebound, except for the baseline level of intrahepatic ccc DNA (4.6 log *vs* 5.4 log, $P = 0.0472$), showing that a lower baseline intrahepatic ccc DNA helps chronic hepatitis patients to maintain a sustained virological response.

As previously reported[15,20], the data obtained from our study show that the reduction in intrahepatic ccc DNA was positively correlated with the change in intrahepatic HBV DNA ($r = 0.525$, $P < 0.05$), and the ccc DNA level was correlated with the serum HBeAg titer at 48 wk after treatment ($r = 0.432$, $P = 0.002$). Since liver tissue is not accessible to obtain for measuring intrahepatic ccc DNA, serum HBeAg titer may be used for assessing the intrahepatic ccc DNA load.

Eight genotypes (A-H) have been found in HBV, each showing a distinct geographical distribution and disease progression. HBV genotype C is believed to be associated with a higher risk of reactivation and progression to cirrhosis compared to HBV genotype B[22,23]. Whether hepatitis B virus (HBV) genotypes influence the response to antiviral treatment remains controversial, and the relationship between HBV genotypes and ccc DNA is still unknown. It was reported that HBV genotypes do not influence the development of resistance to lamivudine, but the severity of liver disease[24]. In the present study, 85.9%

of patients were infected with HBV genotype C and 14.1% with HBV genotype B. There was no significant difference in serum HBV DNA level, intrahepatic HBV DNA and ccc DNA load, as well as in biochemical and histological findings before and after treatment between the two groups. Twenty-four weeks after treatment withdrawal, sustained virological response rate and ALT normalization rate were very similar ($P > 0.05$), indicating that HBV genotypes (B and C) have almost no effect on intrahepatic HBV DNA, ccc DNA load and the long-term efficacy of antiviral therapy.

In conclusion, 48-wk sequential lamivudine - INF-α therapy and lamivudine therapy can significantly reduce HBV DNA and ccc DNA compared to 24-wk INF-α monotherapy. Low baseline intrahepatic ccc DNA level may predict the long-term efficacy of antiviral treatment. Reduction in intrahepatic ccc DNA is positively correlated with the change in intrahepatic HBV DNA, and the ccc DNA level is correlated with the serum HBeAg titer. Intrahepatic ccc DNA level is not significantly affected by the HBV genotype. Since the sample was not large enough, well-designed and placebo-controlled studies are needed.

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COMMENTS

Background

Little effect of antiviral agents on ccc DNA is the main reason for the rebound of HBV DNA after antiviral treatment. It is, therefore, important to measure intrahepatic ccc DNA for monitoring antiviral treatment. The aim of this study was to evaluate the effects of antiviral agents and HBV genotypes on intrahepatic ccc DNA in HBeAg-positive chronic hepatitis B patients.

Research frontiers

After antiviral treatment, relapse occurs in the great majority of chronic hepatitis B patients, and the main reason for the rebound of HBV DNA to its pretreatment level is that the currently available antiviral agents have little effect on ccc DNA (covalently closed circular DNA). Since ccc DNA has been found mainly in liver tissues, how it works or changes due to the action of antiviral agents remains unclear. If it could be taken as a parameter for monitoring antivirus treatment need further study.

Innovations and breakthroughs

It was a first-hand report about the effect of INF-α monotherapy and HBV genotypes on intrahepatic ccc DNA, the relationship between baseline intrahepatic ccc DNA level and the long-efficacy of antiviral treatment.

Applications

Antiviral agents can significantly reduce intrahepatic ccc DNA load, but the duration of treatment should be long enough. Low baseline intrahepatic ccc DNA level may predict the long-term efficacy of antiviral treatment, and can be used as an optimal parameter for monitoring antiviral treatment.

Terminology

ccc DNA (covalently closed circular DNA): After virion enters a susceptible hepatocyte, HBV DNA is then enters the nuclei where it forms a convalently closed circular DNA called ccc DNA. The (-) strand of such a ccc DNA is the template for transcription by cellular RNA polymerase Ⅱ of a longer-than-genome-length RNA called pregenome and a shorter subgenomic transcript, all of which serve as mRNAs.

Peer review

Effects of the currently available antiviral drugs for chronic hepatitis B are not satisfactory. The main reason for the rebound of HBV DNA after therapy is the sustained existence of ccc DNA pool. The aim of this study was to evaluate the effects of antiviral agents and HBV genotypes on intrahepatic ccc DNA in HBeAg-positive chronic hepatitis B patients. The authors found that 48-wk sequential lamivudine - INF-α therapy and lamivudine monotherapy could significantly reduce ccc DNA compared to 24-wk INF-α monotherapy. Low baseline intrahepatic ccc DNA level may predict the long-term efficacy of antiviral treatment and HBV genotypes (C and B).

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RAPID COMMUNICATION

Telomerase-specific oncolytic virotherapy for human hepatocellular carcinoma

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Abstract

AIM: To evaluate the therapeutic efficiency of replicative adenovirus CNHK300 targeted in telomerase-positive hepatocellular carcinoma.

METHODS: CNHK300, ONYX-015 (55 kDa protein deleted adenovirus) and wtAd5 (wild type adenovirus 5) were compared, and virus proliferation assay, cell viability assay, Western blot and fluorescence microscopy were used to evaluate the proliferation and cytolysis selectivity of CNHK300.

RESULTS: The replicative multiples in Hep3B and HepG Ⅱ after 48 h of CNHK300 proliferation were 40625 and 65326 fold, respectively, similar to that of wtAd5.. However, CNHK300 exhibited attenuated replicative ability in normal fibroblast cell line BJ. CNHK300 could lyse hepatocellular carcinoma cells at a low multiplicity of infection (MOI), but could not affect growth of normal cells even at a high MOI.

CONCLUSION: CNHK300 is a cancer-selective replication-competent adenovirus which can cause oncolysis of liver cancer cells as well as wtAd5 (wild type adenovirus 5), but had severely attenuated replicative and cytolytic ability in normal cells. This novel strategy of cancer treatment offers a promising treatment platform.

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Key words: Gene therapy; Virus therapy; Replicative adenovirus; Heptocellular carcinoma

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Li YM, Song ST, Jiang ZF, Zhang Q, Su CQ, Liao GQ, Qu YM, Xie GQ, Li MY, Ge FJ, Qian QJ. Telomerase-specific oncolytic virotherapy for human heptocellular carcinoma. *World J Gastroenterol* 2008; 14(8): 1274-1279 Available from: URL: http://www.wjgnet.com/1007-9327/14/1274.asp DOI: http://dx.doi.org/10.3748/wjg.14.1274

INTRODUCTION

Replication-defective adenoviruses have been successfully established to deliver genes for cancer gene therapy. But, progress in clinical practice has been hampered by lack of cell specific infectivity and poor viral distribution within the tumor mass[1,2]. To confer specificity of infection and increase viral spread in the tumor mass, replication-competent adenovirus is being developed as an promising cancer therapeutic agent. These replication-competent adenoviruses can cause oncolysis of tumor cells as part of the virus life cycle, the new virus progeny released from the dead cells infects the neighboring cells and continues to replicate and spread until the tumor is eradicated. Furthermore, tumor antigens released from the dead cells can enhance antitumor immunity[3]. Until now, more than 10 kinds of replication-competent oncolytic viruses have been evaluated in different phases of clinical trials. This novel strategy of cancer treatment offers a promising treatment platform[4-6].

The key of replication-competent oncolytic adenovirus modification is to constrict viral replication to tumor cells. One approach is to regulate expression of viral genes essential for replication with tumor-specific promoters that are highly active in tumor cells, but inactive in normal cells. For example, human breast cancer has been targeted with the adenovirus Ad. DF3-E1 in which the E1A gene is under the control of the DF3/MUC1 gene promoter/enhancer[7]. Several other tumor-specific promoters are also being evaluated to restrict viral replication to their cognate tumors, such as prostate-specific antigen enhancer element for prostate carcinoma and α-fetoprotein for

hepatocellular carcinoma[8-10]. Although these results demonstrated that these reconstructed adenovirus had high selectivity, their major drawback was that they were only available for treatment of a narrow range of tumors, because only a limited number of tumors could express the targeted tumor markers. Telomerase is the broadest spectrum molecular marker of malignancies, which is highly activated in immortalized cell lines and most of the malignant tumors, but is inactive in normal comatic cells. Human telomerase reverse transcriptase (hTERT) is the major determinant of human telomerase activity[11]. HTERT promoter is highly active in tumor cells but inactive in normal cells, and utilizing hTERT promoter to drive antitumoral genes in cancer gene therapy can target to and selectively kill the cancer cells with positive telomerase activity[12-14].

In this study, we report a strategy for the development of a novel replication-competent oncolytic adenovirus. In this virus, the E1A gene was placed under the control of the human hTERT promoter to allow us to target a wide range of telomerase-positive tumors with little influence on the growth of normal cells.

MATERIALS AND METHODS

Virus

CNHK300 is a tumor-specific replication competent adenovirus variant that was reconstructed at our lab. hTERT gene promoter was inserted at the upstream of the E1A encoding region of the viral genome. Because different cell lines may show various levels of susceptibility to adenovirus infection and virus production, non-selectively replicating wild type 5 adenovirus (wtAd5) was compared with the tested conditionally replicating adenovirus CNHK300. CNHK300-GFP virus carried green fluorescent protein (GFP) as reporter gene which inserted the upstream of hTERT promoter of CNHK300. ONYX-015 is an E1B 55 kDa deleted adenovirus kindly presented by Professor Berk AJ (University of California, USA)[15-17]. All adenoviruses were grown on the human embryonic kidney cell line HEK293. Virus titers were determined by plaque assay. Ratios of virus particle and plaque were between 20:1 and 100:1 normally. CNHK300 and ONYX-015 virus preparation were free of the wtAd5 and endotoxin contamination.

Cells and cell cultures

The human hepatocellular carcinoma cell lines HepG Ⅱ (wild-type p53), Hep3B (mutated p53) and human normal fibroblast cell line BJ were purchased from the American Type Culture Collection (Manassas, VA). Transformed human embryonal kidney HEK293 cell line was obtained from Microbix Biosystem Inc. (Toronto, Ontario, Canada). HEK293, HepG Ⅱ and Hep3B were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone). BJ was maintained in Minimum Eagle Medium (MEM) with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine. All the media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin and maintained at 37℃ in 5% CO_2.

Detection of hTERT mRNA in various cell lines

RT-PCR amplification method was used to detect the hTERT mRNA. Total RNA was extracted from cell lines by the Trizol Reagent (Invitrogen Corp., San Diego, CA). First strand cDNAs synthesis and PCR reaction were performed using the SuperScript one-step RT-PCR with Platinum Taq Kit (Invitrogen Corp.) according to the manufacturer's protocol. The reaction mixture was heated at 50℃ for 30 min, and then 30 cycles of PCR were performed, including denaturation at 94℃ for 30 s, annealing at 60℃ for 30 s and extension at 72℃ for 60 s, and then 72℃ for 2 min. For PCR reaction, sense and antisense oligonucleotide primers (5'-CGGAAGAGT GTCTGGAGCAA-3'; 5'-GGATGAAGCGGAGTC TGGA-3') were designed. The PCR products (150 bp in length) were electrophoresed on 10% agarose gel.

Detection of E1A expression by Western blotting

HepG Ⅱ, Hep3B and BJ cells were plated at a density of 5×10^5 in 6-well plates (Falcon) and 24 h later infected with CNHK300, ONYX-015 or wtAd5 at a MOI corresponding to IC_{50}. After a 2 h incubation at 37℃ in 5% CO_2, culture medium were removed, the cells were then washed twice with serum-free medium and incubated at 37℃ in 5% CO_2. Forty-eight hours later, culture medium were removed, total cell lysates were prepared using 150 μL/well of Mammalian Protein Extraction Reagent (M-PER, PIERCE, Rockford, IL). Twenty μL of aliquots were subjected to detection on 10% SDS-PAGE and E1A protein was detected with M73 antibody against adenovirus E1A (Santa Cruz Biotechnology, Santa Cruz, CA). The signals were visualized with LumiGLO chemiluminescent reagent and peroxide (Cell Signaling Technology, Bevery, MA).

Proliferation of CNHK300-GFP and GFP expression

Hep3B and BJ cells were plated at a density of 2×10^5 in 6-well plates (Falcon). When the cells reached 80% confluence 2-4 d later, the cells were infected with CNHK300-GFP at a MOI of 0.01, 1, 10 plaque forming unit (pfu)/cell, respectively. The cells were observed and photographed at 3, 7 and 10 d after infection.

In vitro viral replication assay in different cell types

HepG Ⅱ, Hep3B and BJ cells were plated at a density of 5×10^5 in 6-well plates (Falcon) and 24 h later infected with CNHK300, ONYX-015 or wtAd5 at MOI of 5 pfu/cell. After a 2 h incubation at 37℃ in 5% CO_2, culture medium were removed, the cells were then washed twice with serum-free medium and incubated at 37℃ in 5% CO_2 for varying periods of time (0 h, 12 h, 24 h, 48 h, 96 h), the cultures at each time point were harvested and lysed by three cycles of freeze and thaw. The supernatant of each time point was tested for virus production on 293 cells for 10 d with plaque assay. The relative ability of the viruses to amplify in these cells after infection was calculated by comparing the ratio of viral yields to initial virus input.

In vitro cell viability assay

MTT assay was performed to determine cell viability at

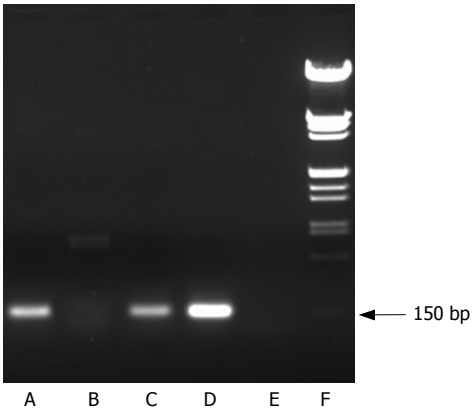


Figure 1 CNHK300 hTERT mRNA expression in various cell lines. Telomerase hTERT mRNA expression was positive in cancer cell lines HepG Ⅱ (A) and Hep3B (C), but not in the normal fibroblast cell line BJ (B) CNHK300 hTERT mRNA expression was detected in E1 transformed human embryonal kidney cell line HEK293 (D) as a positve control. E: Negative control, F: DNA marker.

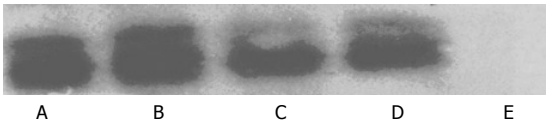


Figure 2 Detection of E1A expression by Western blotting. CNHK300 could express E1A in HepG Ⅱ (A) and Hep3B (C) cells but not in the BJ human normal fibroblast cells (E), whereas wtAd5 did not show any selectivity in E1A expression, wtAd5 E1A expression was positive in Hep3B cells (D). E1A expression was detected in E1 transformed human embryonic kidney cell line HEK293 (B) as a positive control.

various viral MOIs. HepG Ⅱ, Hep3B and BJ cells were plated at a density of 1×10^4 in 96-well plates (Falcon) and 24 h later, the cells were infected with CNHK300 at increasing MOIs. After 7 d of incubation, cell viability was measured by the MTT assay using the non-radioactive cell proliferation kit (Roche Molecular Biochemicals) according to the kit protocol and the spectrophotometrical absorbance of the samples using a microtiter plate (ELISA) reader at 570 nm (the reference wavelength was 650 nm). The percentage of cell survival was calculated using the formula: Cell survival (%) = (A value of infected cells/A value of uninfected control cell) × 100%. Eight replicate samples were taken at each MOIs, and each experiment was performed at least three times.

RESULTS

Detection of hTERT mRNA in various cell lines

RT-PCR was performed to detect the hTERT mRNA expression in HepG Ⅱ, Hep3B cells and normal cells. As shown in Figure 1, telomerase hTERT mRNA expression was demonstrated in both cancer cells tested. In contrast, BJ fibroblast cells did not express hTERT mRNA.

Detection of E1A expression by Western blotting

The Western blotting revealed that CNHK300 could express E1A in the HepG Ⅱ and Hep3B cells but not in the BJ human normal fibroblast cells. As controls, BJ cells were also infected with wtAd5, and the results indicated that wtAd5 could effectively express E1A in BJ cells. These findings demonstrated that CNHK300 selectively expressed E1A in telomerase positive hepatocellular cancer cells, whereas wtAd5 did not show any selectivity in E1A expression (Figure 2).

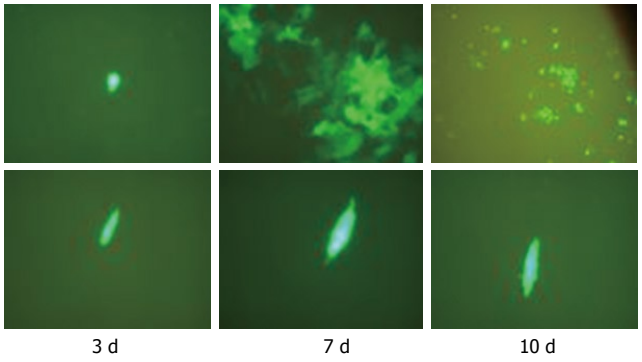


Figure 3 Proliferation selectivity of CNHK300-GFP in Hep3B (upper) and BJ (lower) under fluorescence microscope. CNHK300-GFP could infect both BJ cells and Hep3B cells. But after days of infection, CNHK300-GFP virus could proliferate effectively in cancer cells and induced cytopathologic effect (CPE) at d 7, whereas in normal cell lines, CNHK300-GFP proliferation was attenuated, and BJ cells did not show CPE after days of infection.

Proliferative selectivity of CNHK300-GFP

As shown in Figure 3, CNHK300-GFP could infect both BJ and Hep3B cells and expressed GFP within 3 d. But after 7 d of infection, CNHK300-GFP virus could proliferate effectively and express GFP protein. When the CNHK300-GFP was released from the infected cancer cells, it could infect the adjacent cells and enter into new life cycles. Therefore, the cancer cells showed obvious proliferation and cytopathologic effect (CPE) at the 7th day. Because many cancer cells died from virus infection after 10 d of infection, the GFP protein was degraded and extincted gradually. Whereas in normal cell lines, CNHK300-GFP proliferation was attenuated, and BJ cells did not show CPE after days of infection (Figure 3).

Replication of CNHK300 attenuated in normal cells but not in tumor cells

To determine whether the CNHK300 viruses we reconstructed replicate preferentially in tumor cells and replicatively attenuated in normal cells, we performed virus yield assay in HepG Ⅱ and Hep3B tumor cell lines and BJ normal cell lines, and because different cell lines may show different susceptibility to adenovirus infection and replication ability to correct the difference in infectivity and virus production between various tumor cell lines and normal cell lines, wtAd5 served as a control. As shown in Figure 4, CNHK300 replicated by 40 625 and 65 326 folds on tumor cell HepG Ⅱ and Hep3B, respectively, similar to those of wtAd5. However, the CNHK300 replication (180-folds) was attenuated significantly as compared with wtAd5 (4000-folds) in BJ normal cell lines.

Specificity of cytotoxicity of CNHK300 by MTT assay

MTT assay was performed to characterize the specificity

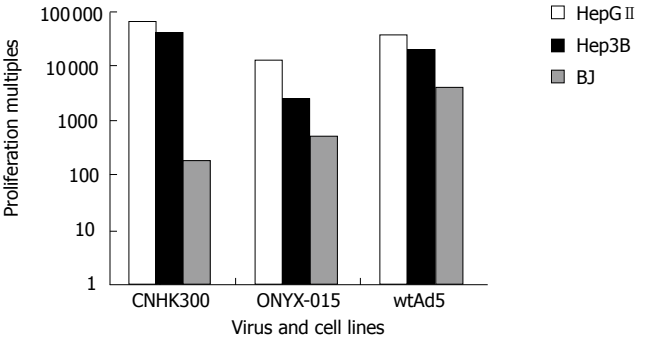


Figure 4 CNHK300, ONYX-015 and wtAd5 proliferation multiples in hepatocellular cancer cells HepG Ⅱ, Hep3B and normal fibroblast cells BJ after 48 h virus infection. CNHK300 replicated by 40 625 and 65 326 folds on tumor cell HepGII and Hep3B, similar to those of wtAd5. The CNHK300 replication (180-folds) was attenuated significantly as compared with wtAd5 (4000-folds) in BJ normal cell lines.

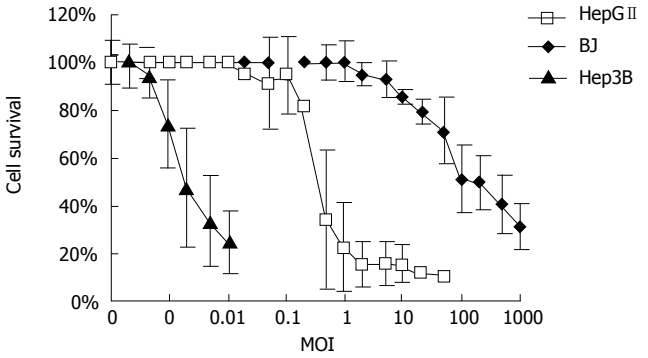


Figure 5 Hepatocellular cancer cells HepGII, Hep3B and normal fibroblast cells BJ survival curves after the cells were infected with CNHK300 at increasing MOIs. CNHK300 caused significant cytolysis in HepGII and Hep3B tumor cell lines with a MOI of 0.5 pfu/cell and 0.0002 pfu/cell. For normal fibroblast cell lines BJ in contrast, cells infected with CNHK300 showed over 50% cell viability rate at the same time points with MOI of 100 pfu/cell.

of CNHK300 on tumor cells with no or less toxicity than normal cells. As shown in Figure 5, CNHK300 could cause significant cytolysis in HepG Ⅱ and Hep3B tumor cell lines with a MOI of 0.5 pfu/cell and 0.0002 pfu/cell, respectively. However, cells infected with CNHK300 showed an over 50% cell viability rate at the same time points with MOI of 100 pfu/cell, suggesting that more than 200-500 000 folds of CNHK300 were needed to kill half of normal fibroblast cells compared with HepG Ⅱ and Hep3B tumor cells.

DISCUSSION

Many independent studies indicated that telomerase was active in over 85% malignant cancers, but not in normal tissues except hematopoietic stem cells and germ cells in the ovary and testis. Telomerase activation has been regarded as the critical characteristic of malignant tumors[18]. hTERT is the major determinant of human telomerase activity, and hTERT promoter has been successfully used to drive antitumoral genes in cancer gene therapy to target to and selectively kill the cancer cells with positive telomerase activity[19-21]. In our study, we constructed a replicative adenovirus CNHK300, in which, the E1A gene was placed under the control of the human hTERT promoter to allow us to target a wide range of telomerase-positive tumors[22]. A key requirement for clinical application of tumor-specific oncolytic virus is their attenuation in non-target tissues. So, *in vitro* viral replication assay and *in vitro* cell viability MTT assay were used to test the replicative specificity and cytotoxicity of CNHK300 in cancer cell lines and attenuated cytolysis in normal cells. Our experiments achieved similar results with some foreign studies[23-25]. As shown in our results, CNHK300 showed strong differences in replication and cytotoxicity in different hepatocellular cell lines. Though we tested higher titers of CNHK300 in HepG Ⅱ cells than in Hep3B cells after 48 h of virus exposure, CNHK300 did not show stonger cytotoxicity on HepG Ⅱ cells. There might be some other cell-killing mechanisms besides oncolytic effect due to virus replication.

Hematopoietic stem cells and germ cells in the ovary and testis are all telomerase-positive cells. Therefore, CNHK300 may replicate in above normal cells if it infects these cells. Fortunately, CNHK300 can not get into these cells because they lack adenovirus-specific CAR receptor on their cell surface[26].

Adenovirus is widely used in generating replication-selective viruses. In recent years, two major strategies have come into force. In the first one, viral genes that become dispensable in tumor cells, such as the genes responsible for activating the cell cycle through p53 or Rb binding, have been completely or partially deleted[15-17]. E1b 55 kDa deleted adenovirus Onyx-015 was proposed to replicate selectively in p53-deficient cells. Yet, several studies indicated that Onyx-015 was independent of the p53 status[15-17]. In the second strategy, transcription of viral genes has been controlled by replacing the native viral promoters with tumor-specific promoters[8-10,22-25]. In contrast, great progress has been made in the study of tumor-specific promoter regulated replication-selective adenovirus, Paul Hallenbeck (Genetic Therapy-Novartis, Gaithersburg, MD) and Daniel R. Henderson (Calydon, Sunnyvale, CA) have initiated the efforts in this direction using the α-fetoprotein (AFP) and prostate specific antigen (PSA) promoters to drive the adenovirus E1A gene. It has been proved that modified adenovirus using this strategy has some advantages compared with Onyx-015[27-30].

COMMENTS

Background

Viruses can infect, replicate in and kill human cells through diverse mechanisms. Clinicians have treated hundreds of cancer patients with a wide variety of wild-type viruses over the last century, but the approach was abandoned due to toxicity. However, with the advance of recombinant DNA technology, it became possible to genetically engineer viruses to enhance their safety and antitumoral potency. Conditionally replicating adenoviruses (CRAds) represent a promising new platform for the treatment of malignant tumors. Recently, the promoter of human telomerase reverse transcriptase (hTERT) was used to restrict adenoviral replication to telomerase-positive cancer cells through controlling the adenoviral E1a gene. Compared with other promoters, the hTERT promoter is highly active in more than 85% of different human cancers, but inactive in most normal somatic cells, therefore. this mechanism can be applied to a wide range of cancers. In this study, the authors tested the antitumor activity of the hTERT promoter regulated tumor-selective RCAd CNHK300, and confirmed its highly efficient antitumor activity *in vitro* in HCC cell lines.

Research frontiers

In recent years, two major strategies have come into force. In the first one, viral genes that become dispensable in tumor cells, such as the genes responsible for activating the cell cycle through p53 or Rb binding, have been completely or partially deleted. In the second strategy, transcription of viral genes has been controlled by replacing the native viral promoters with tumor-specific promoters. McCormick's group at Onyx proposed that an E1b 55 kDa deleted adenovirus Onyx-015 would replicate selectively in p53-deficient cells. Recently, the progress made in the study of tumor-specific promoter regulated replication-selective adenovirus has proved that modified adenovirus using this strategy has some advantages compared with Onyx-015.

Innovations and breakthroughs

Tumor specificity is a major challenge in viral therapy. For adenovirus, one strategy is the development of promoter-driven replicative adenovirus to achieve selective-replication adenovirus. The promoters of AFP, MUC-1 and PSA have been studied to control the adenovirus E1A expression targeting hepatocellular, breast and prostate carcinomas, respectively. In CNHK300, the E1A gene was placed under the control of the human hTERT promoter to allow us to target a wide range of telomerase-positive tumors with little influence on the growth of normal cells.

Applications

The results demonstrated CNHK300 could replicate in liver cancer cells efficiently as well as wtAd5 and kill heptocellular carcinoma, but had severely attenuated proliferation and cytolysis in normal cells. It may has a strong potential for targeting therapy of a wide range of cancers including hepatocellular carcinoma.

Peer review

This is a paper worthy of publishing. The design of the study in all aspects is methodologically sound. hTERT promoter-regulated replicative adenovirus CNHK300 can specifically kill a wide range of cancer cells including HCC. This new cancer therapy strategy may be proven to be very promising in clinical practice.

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RAPID COMMUNICATION

Development of a survival evaluation model for liver transplant recipients with hepatocellular carcinoma secondary to hepatitis B

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Abstract

AIM: To develop a model using easily obtainable, objective, verifiable preoperative parameters, to help evaluate post transplant survival probability for hepatocellular carcinoma (HCC) patients with hepatitis B.

METHODS: We retrospectively examined a cohort of 150 consecutive primary cadaveric liver transplants with HCC in our center over 6 years. Thirteen preoperative biochemical parameters and six tumor-related factors were analyzed to identify their correlation with post transplant survival using the Cox proportional-hazards regression model. The predictive power of a new model and the model for end stage liver disease was compared by the receiver operating characteristic curve.

RESULTS: In univariate analysis, the factors significantly associated with post transplant survival were serum concentrations of albumin, total bilirubin, alkaline phosphatase, alpha-fetoprotein, γ-glutamyltransferase, aspartate aminotransferase, sodium, tumor diameter and the number of tumor nodules. Multivariate analysis showed alpha-fetoprotein, serum sodium, alkaline phosphatase and the number of tumor nodules were significantly associated with the post transplant outcome. Based on the four variables, we established a new model with a c-statistic of 0.72 which was significantly greater than 0.50 (P = 0.001), and the c-statistic of MELD was 0.59 (P = 0.146).

CONCLUSION: The new model based on four objective tumor-related parameters has the capacity to evaluate the risk of post transplant mortality for HCC patients with hepatitis B.

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Key words: Liver neoplasms; Hepatitis B; Liver transplantation; Survival evaluation; Model for End Stage Liver Disease

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INTRODUCTION

As a high hepatitis risk region, hepatocellular carcinoma (HCC) patients in China account for approximately 55% of HCC over the world, and more than 90% HCC in China are related to hepatitis B and accompanied by cirrhosis[1]. Liver transplantation (LT) has become accepted as an effective therapeutic modality for these patients[2,3], but the discrepancy between the livers available and patients in need of transplantation is outstanding. In 2002, the new MELD-based allocation policy was implemented by the United Network of Organ Sharing (UNOS) as the criteria for liver allocation[4]. Although the model for end stage liver disease (MELD) has been validated to predict disease severity and ranking in LT candidates on the waiting list[5-7], its capacity to predict post-LT outcome remains controversial. Heretofore, the relationship between severity of illness at LT and outcome after LT is still a major area of debate[8], especially for HCC candidates.

MELD was developed based on benign liver diseases and many aspects of malignant tumor were not taken into

account[9], therefore, the proposal to use the MELD system for post transplant evaluation lacks predictive power. Some studies supported that HCC-related parameters should be added into MELD to improve its assessing capacity for the HCC patients[10]. The aim of our study, therefore, is to find some risk factors associated with post transplant outcome of HCC recipients and attempt to develop a new model using some easily obtainable, objective, verifiable preoperative parameters, to help assess post-LT survival probability.

MATERIALS AND METHODS

Subjects

We followed 161 consecutive HCC patients aged 18 years or older who underwent primary cadaveric LT at West China Liver Transplantation Center between February 1999 and June 2006. All of the diagnoses of HCC were confirmed by pathology. Among these 161 recipients, eight were eliminated because of failure in follow-up; three, for death within 10 d after transplantation due to some perioperative factors. The remaining 150 transplants were under analysis. All included recipients' demographic characteristics, pre-LT laboratory data and HCC-related parameters were abstracted from our liver transplant database (Tables 1, 2 and 3), and all data were from the latest examination before LT. Just as the radiological Milan criteria, the tumor features in our study were also obtained by CT or MRI, and all last images were within 3 d before transplantation. The same surgical team performed all LTs using standard techniques, and all recipients were followed up by June 30, 2007.

MELD calculation

The MELD scores were calculated according to the following model[5]: MELD = 3.78 × $\log_e$TB (mg/dL) + 11.20 × $\log_e$INR + 9.57 × $\log_e$Crea (mg/dL) + 6.4. In this formula, values for creatinine, INR and TB less than 1.0 were reset to equal 1, and values for creatinine were capped at 4. In patients with creatinine value less than 4 who were on dialysis at the time of transplantation, the creatinine should be increased to equal 4.

Statistical analysis

Cox proportional-hazards regression was the main statistical tool for survival modeling. Recipients' survival was estimated by the Kaplan-Meier method, and comparison of survival differences performed by the log-rank test. To decrease the influence of extreme laboratory values, all quantitative variables were transformed to their natural logarithms ($\log_e$ value)[5]. We first performed univariate Cox regression analysis of all parameters shown in Tables 2 and 3, and those found to be significant at the 0.10 level were selected for the multivariate analysis. The final multivariate model was obtained by a backward elimination stepwise selection method with the criteria for factor removal being $P \geqslant 0.05$. Based on the selected variables, prognostic index (PI) and survival function could be constructed.

To assess the validity in predicting the risk of post transplant death, we performed ROC curves for the two models and measured their concordance statistic (c-statistic), the mathematical measure to determine the validity of a model[11,12]. Comparison of the c-statistic was done using the method of Hanley[13]. All analyses were performed using SPSS13.0 statistical software.

Table 1 Demographic characteristics of recipients

Gender	*n* (%)
Male	129 (86.0)
Female	21 (14.0)
Median age (range)	51 (28-68)
Hepatitis B	150 (100)
Child's score	
A (5-6)	89 (59.3)
B (7-9)	46 (30.7)
C (10-15)	15 (10.0)
MELD score	
⩽ 18	132 (88.0)
> 18	18 (12.0)

Table 2 Pretransplant biochemical features of recipients

Variables	mean ± SD	Min value	Max value
Hb (g/dL)	12.23 ± 2.62	4.3	18.7
PLT (× 10^9)	131.4 ± 86.0	17	569
Urea (mmol/L)	4.90 ± 2.01	2.4	16.2
Crea (mg/dL)	1.06 ± 0.10	1	1.44
ALB (g/dL)	3.79 ± 0.68	2.01	5.28
TB (mg/dL)	26.8 ± 37.4	10	260.2
AKP (u/L)	160.51 ± 130.97	18	779
GGT (u/L)	215.34 ± 255.40	24	1630
AST (u/L)	89.83 ± 76.23	19	421
ALT (u/L)	101.49 ± 99.19	12	486
INR for PT	1.25 ± 0.24	1	2.16
AFP (μg/L)	426.65 ± 393.91	1.97	3000
Na^+ (mEq/L)	137.12 ± 5.33	114.2	150.3

RESULTS

Univariate analysis

Table 4 shows the results of the univariate analyses: serum concentration of ALB, TB, AKP, GGT, AST, AFP, Na^+, tumor diameter and the number of tumor nodules were significantly associated with survival after transplantation. The positive regression coefficient implies that the risk of death would increase with increasing values of a risk factor.

Multivariate analysis

Of the candidate variables derived from univariate analyses, serum concentration of AKP, AFP, Na^+ and the number of tumor nodules were significant predictors of survival after transplantation (Table 5). The negative regression coefficients of "Na^+" and "single tumor nodule" imply that the risk of death would decrease with their increasing values.

Calculating the prognostic index and predicted survival probabilities

The prognostic index for individual patients can be

Table 3 HCC-related features of recipients

Variable		Code	Frequency	Proportion (%)
History of hepatectomy for HCC	Present	1	15	10
	Absent	0	135	90
Tumor diameter	1: < 5 cm	1	65	43.3
	2: 5 cm-	2	58	38.7
	Reference: > 10 cm	3	27	18
Tumor nodule	Nodule 1: one	1	93	62
	Nodule 2: two	2	31	20.7
	Reference: > two	3	26	17.3
Vascular invasion	Present	1	20	13.3
	Absent	0	130	86.7
Intrahepatic tumor dissemination	Present	1	33	22
	Absent	0	117	78
Perihepatic lymphadenectasis	Present	1	10	6.7
	Absent	0	140	93.3

calculated by combining their four prognostic values with the regression coefficients reported in Table 5. The formula for PI as well as the MELD is given as follow:

PI = 0.663 × $\log_e$ (AKP u/L) + 0.122 × $\log_e$ (AFP μg/L) - 8.755 × $\log_e$ (Na^+ mEq/L) - 0.631 × the number of tumor nodules (single nodule coded 1, multinodular tumor coded 0).

To assess the probability of survival at a certain time after LT, we constructed the survival function of the new model[9,14]: $S(t) = [S_0(t)]^P$ P = expPI. $S(t)$ was the survival function, and $S_0(t)$ was the individual baseline survival function which could be calculated using the linear interpolation method in baseline survival rate series. Finally, the survival probability $S(t = \chi)$ of time point χ could be computed by putting both the PI value and the $S_0(t = \chi)$ into the above formula. The larger the $S(t)$ is, the higher probability of death of the patient would be after time point χ.

Validation of the new model and comparison with MELD

The recipients' post transplant survival was compared based on this new model. Because their PI values did not follow the normal distribution (*Shapiro-Wilk* test, $W = 0.949$, $P = 0.001$), recipients were stratified into two risk groups according to the median of PI values, namely a high PI value (high risk) group and a low PI value (low risk) group. The low risk group had a better prognosis than the high risk group, and the survival difference between them was statistically significant (*log-rank* test, $\chi^2 = 10.71$, $P = 0.001$ Figure 1A).

According to ROC analysis (Figure 1B), the c-statistic of the new model was 0.72 (95% CI = 0.609-0.824) which was significantly greater than 0.50 ($P = 0.001$), and the c-statistic of MELD was only 0.59 (95% CI = 0.470-0.711) which did not have significantly predictive value ($P = 0.146$). The c-statistic may range from 0 to 1, with 1 indicating perfect discrimination and 0.5 (the area under the chance line) indicating what is expected by chance only[15]. When a c-statistic above 0.50 has statistical significance compared with 0.50, it means the model is valid[9]. Meanwhile, for the prognostic model, with a c-statistic above 0.7 is generally considered as a useful model[5], and a c-statistic below 0.7 suggests poorly predictive ability[16]. So the new model was valuable in assessing the post transplant outcome.

Table 4 Univariate Cox analysis of parameters

Variable	Regression coefficient	Regression coefficient SE	Wald	*P*-value	Exp$^\beta$
Hb ($\log_e$)	-0.510	0.510	0.997	0.318	0.601
PLT ($\log_e$)	0.060	0.230	0.068	0.795	1.062
Urea ($\log_e$)	0.024	0.457	0.003	0.958	1.025
Crea ($\log_e$)	-0.427	1.671	0.065	0.798	0.652
ALB ($\log_e$)	-2.056	0.695	8.758	0.003	0.128
TB ($\log_e$)	0.277	0.165	2.838	0.092	1.320
AKP ($\log_e$)	0.581	0.214	7.328	0.007	1.787
GGT ($\log_e$)	0.254	0.148	2.930	0.087	1.289
AST ($\log_e$)	0.535	0.202	6.987	0.008	1.707
ALT ($\log_e$)	-0.172	0.178	0.926	0.336	0.842
INR ($\log_e$)	-0.065	0.818	0.006	0.937	0.937
AFP ($\log_e$)	0.104	0.071	2.742	0.098	1.109
Na^+ ($\log_e$)	-7.161	3.144	5.190	0.023	0.001
History of hepatectomy	-0.160	0.474	0.115	0.735	0.852
Tumor diameter			6.245	0.089	
1	-1.057	0.505	4.385	0.036	0.347
2	0.278	0.359	0.603	0.437	1.321
Tumor nodule			5.410	0.067	
1	-0.391	0.364	1.153	0.283	0.677
2	0.419	0.419	1.000	0.317	1.520
Vascular invasion	0.368	0.300	1.503	0.22	1.445
Intrahepatic tumor dissemination	0.190	0.345	0.305	0.581	1.210
Perihepatic lymphadenectasis	0.583	0.526	1.226	0.268	1.791

Table 5 Multivariate Cox assessment of risk factors associated with post-LT mortality

Variable	Regression coefficient	Regression coefficient SE	Wald	*P*-value	Exp$^\beta$
AKP ($\log_e$)	0.663	0.222	8.917	0.003	1.941
AFP ($\log_e$)	0.122	0.074	4.042	0.048	1.130
Na^+ ($\log_e$)	-8.755	3.252	7.247	0.007	0.000
Tumor nodule			8.189	0.017	0.532
1	-0.631	0.373	4.265	0.041	

DISCUSSION

The MELD has been validated to predict the pretransplant mortality risk in patients with many types of end-stage liver disease and to determine the medical urgency for transplantation[5,7,8], but abundant further investigations indicate it's not adequate for HCC candidates[4,17]. We hold that the main reason could be attributed to the fact that the development of MELD was based on benign liver diseases and did not incorporate aspects of malignant tumors[9]. Therefore, modifications were made in the MELD/HCC allocation system in April 2003[18]. Meanwhile, although the value of MELD for assessing disease severity and predicting pretransplant survival has been convincingly established, its ability to evaluate post transplant outcome is still under controversy. To date, many studies have proved the poor capacity of MELD in predicting post

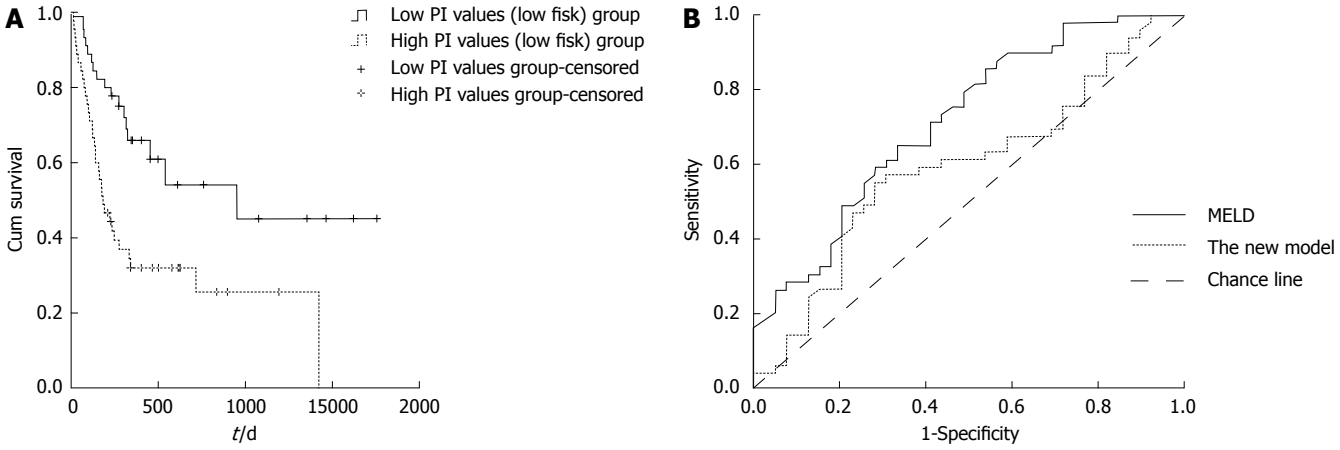


Figure 1 **A**: Comparison of posttransplant survival (Kaplan-Meier) for two groups of recipients according to the median of their PI values; **B**: Receiver operating characteristic curve (ROC) for MELD and the new model.

transplant survival[19-22]. We also confirmed the MELD scores were poorly correlated with the post transplant prognosis of HCC recipients, its c-statistic was only 0.59.

A model like the MELD which can stratify disease severity may optimize the timing of LT, and a prognostic model which can predict the post transplant survival probability may screen the appropriate candidates who can benefit mostly from the transplantation. The importance of understanding predictors of post-LT outcome before surgery was first recognized in 1987 by Malatack[23], who identified several pretransplant parameters associated with post transplant prognosis in pediatric recipients. Similarly, we also identified four variables correlated with post transplant outcome of HCC/LT recipients in our study: the serum concentration of AKP, AFP, Na^+ and the number of tumor nodules, which were easily available, objective and reproducible biochemical tests and tumor-related factors before operation. Further analyses confirmed this newly derived model based on these four variables resulting in a c-statistic of 0.72, which indicates its validity in post transplant outcome evaluation. We consider that the inclusion of these tumor-related parameters may contribute to the validity of our model because of the close correlativity between cancer biological features and patients' prognosis.

The serum concentration of AFP, combining with specific imaging techniques, has been one of the diagnostic criteria for HCC on account of its satisfactory sensitivity and specificity[24]. Also, AFP level is an important predictor of HCC prognosis[25]. The pretransplant AFP level was proved to associate with tumor recurrence and prognosis[26], and the adjuvant treatments often fail to prevent tumor progression in patients with high AFP level[27]. Ochiai *et al*[28] investigated 95 HCC patients undergoing hepatectomy and found that the serum AFP concentration above 400 μg/L was one of the preoperative risk factors associated with postoperative prognosis. Furthermore, studies from Carlis *et al*[29] and Del Gaudio *et al*[30], who retrospectively analyzed 121 and 87 HCC patients who received LT, verified serum AFP level was not only significantly related to the development of tumor recurrence, but also a significant independent risk factor of patient overall-survival after transplantation. Our finding was consistent with these studies which all identified AFP level did associate with the postoperative outcome.

Liver is a main organ to synthesize and secrete AKP in adults. Pathological changes of the hepatocytes, therefore, will cause certain changes of AKP, so it is a parameter to indicate the hepatic function. Meanwhile, since the elevated serum concentration of AKP can be observed in several liver malignant tumors such as intrahepatic cholangiocellular carcinoma and HCC, it has also been accepted as a kind of tumor marker even though its specificity is not so satisfactory yet. Screening by mathematical statistics in our study revealed that combination of AFP and AKP could have predictive value for HCC recipients in post-LT survival evaluation. If the serum concentration of AFP and AKP stay at a high level continuously or increase again after transplantation, it is usually parallel to the presence of residual tumor cells or the probability of cancer recurrence. That is consistent with what we observed clinically.

MELD does not encompass complications of portal hypertension, but many studies suggest some complications, especially the refractory ascites and serum sodium are predictors of mortality for patients with liver diseases[31-34]. In 2004, a preliminary report from Argentina first documented that addition of serum sodium to the MELD as either a dichotomous or a continuous variable could improve its predictive power[35]. Further, studies from Heuman *et al*[31] and Biggins *et al*[32] confirmed serum sodium could enhance MELD and help risk-stratify patients more accurately. The correlation between ascites and hyponatremia had been already documented by several reports[6,31]. Compared to the serum sodium, ascites is not consistently quantified, and standardization of ascites measurement among centers cannot be easily accomplished because it's a subjective and ambiguous variable. According to abundant evidence-based proofs, a conference in 2004 recognized this fact and recommended collection of serum sodium for all liver registrants to ascertain the value of adding this parameter into MELD[36]. The UNOS/OPTN Board also approved inclusion of serum sodium, either as

a dichotomous or as a continuous variable, to the list of laboratory tests collected in liver transplant recipients[36].

In our study, serum sodium showed significant association with post transplant prognosis. Its prognostic value may be reflected by the way that serum sodium can characterize patients with the hemodynamic or internal environmental derangement and the poor general conditions. Its negative regression coefficient indicates that the effect of serum sodium in whole model is protective, which means the higher the serum sodium concentration is, the lower risk of mortality the patient would be.

The number of tumor nodules is a specific character for tumor patients and a well-recognized predictor of prognosis in patients with HCC. Various clinical and pathologic factors related to the cancer recurrence and recipients' outcome have been validated in many studies, including the tumor size, the presence of satellite nodules, multilobarly distributed tumors, intrahepatic or extrahepatic dissemination, vascular invasion (microscopic and macroscopic), histological degree of differentiation[10,30,37,38]. Our results identified the number of tumor nodule as an independently predictive variable associated with post transplant survival, which was consistent with the findings from Bismuth[37]. Not surprisingly, either the tumor development from multicenter or the presence of intrahepatic metastasis will apparently parallel the poor prognosis in HCC recipients. That's why the selection guidelines for HCC candidates which have been generally acknowledged and widely used, such as the Milan criteria and the UCSF criteria, not only pay attention to the tumor size, but have severe restrictions to the number of tumor nodules.

The results of our study showed the new model, which employs four objective and widely reproducible variables, were more useful in assessing the prognosis of HCC recipients than MELD. Our study supported that incorporation of related biochemical parameters and tumor-specific variables would be a feasible way to develop a model for post transplant outcome assessment in HCC recipients. This kind of prognostic model, we believe, should play an important role in the selection of the best appropriate HCC candidates into liver allocation schemes. Whether the new model is widely useful or not, still needs to be validated by further investigation. We hope there would be continued improvement of the model, and we are looking forward to the confirmation of our findings in larger-volume patients' series.

COMMENTS

Background

Liver transplantation has been accepted as an effective therapeutic modality for HCC patients, but the relationship between severity of illness at LT and outcome after LT is still a major area of debate. Although the MELD has been proved to accurately predict pretransplant mortality, it's still controversial whether the MELD can evaluate post transplant outcome for HCC recipients.

Research frontiers

Although the value of MELD for assessing disease severity and predicting pretransplant survival has been convincingly established, abundant further investigations proved its poor capacity in predicting post transplant survival. Many studies have supported the HCC-related parameters should be added into MELD to improve its assessing capacity for the HCC patients.

Innovations and breakthroughs

In this study, we developed a new model to evaluate the risk of post transplant mortality in recipient of liver transplantation for HCC with hepatitis B. Our results also confirmed that this new model based on the four objective tumor-related parameters is more useful than MELD in assessing the prognosis of HCC recipients.

Applications

A prognostic model which can evaluate the risk of post transplant mortality may screen the appropriate candidates who can benefit mostly from the transplantation. So the new model, we believe, could play some role in selection of the best appropriate HCC candidates into liver allocation schemes.

Peer review

This paper is of some interest, its results support that incorporation of related biochemical parameters and tumor-specific variables would be a feasible way to develop prognostic model for post transplant outcome assessment in HCC recipients. This idea is deserved to explore.

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RAPID COMMUNICATION

Relationship between the cytotoxin-associated gene-A status of *H pylori* strains and cerebral infarction in European Caucasians and Chinese Han: A meta-analysis

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Abstract

AIM: To study the relationship between the cytotoxin-associated gene-A (CagA) status of *H pylori* strains and cerebral infarction among European Caucasians and Chinese Han by conducting a meta-analysis.

METHODS: Ten case-control studies, with data on a total of 907 cases and 966 controls, were retrieved and considered; disqualified studies were excluded. The included studies were then tested for heterogeneity, and a meta-analysis was performed.

RESULTS: The combined data revealed CagA-bearing strains of *H pylori* which cause chronic infection are associated with an increased risk of cerebral infarction (OR = 2.66, 95% CI: 2.17-3.26), but no such relationship was found with CagA-negative strains (OR = 0.74, 95% CI: 0.49-1.10) in the overall population. We performed subgroup analyses, dividing the overall population into European Caucasians and Chinese Han subgroups, and analyzed the studies according to their subgroup classification. Through the subgroup analysis, an association between cerebral infarction and CagA-bearing strains was found in both subgroups (OR = 2.60, 95% CI: 1.93-3.49 in Chinese Han; OR = 2.71, 95% CI: 2.05-3.59 in European Caucasians), but no significant association was found between cerebral infarction and CagA-negative strains (OR = 0.81, 95% CI: 0.45-1.48 in Chinese Han; OR = 0.64, 95% CI: 0.37-1.09 in European Caucasians).

CONCLUSION: These results suggest CagA-bearing strains of *H pylori* are significantly associated with susceptibility to cerebral infarction in Chinese Han and European Caucasians, but that CagA-negative strains are not a definite predisposing factor in either subgroup. The magnitude of this association with cerebral infarction needs to be confirmed by prospective studies and combined studies of *H pylori* eradication.

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Key words: Cytotoxin-associated gene-A; *H pylori*; Cerebral infarction; Meta-analysis

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INTRODUCTION

There is increasing evidence, from both clinical and experimental observations, that chronic inflammation (including that caused by Chlamydia pneumoniae, Cytomegalovirus and *H pylori*) plays an important role in the development of atherosclerotic lesions in patients with coronary artery disease and ischemic stroke. The role of chronic inflammation in atherogenesis has been studied extensively over the past decade. Conceiving atherosclerosis as an inflammatory condition has given rise to a series of hypotheses aimed at finding determinants for the occurrence of cardiovascular and cerebrovascular diseases beyond the traditional risk factors[1]. Among these hypotheses, the contributions of chronic infections to the development of atherothrombosis have been investigated in many cross-sectional and prospective studies, and are currently regarded as co-operating factors in maintaining a chronic inflammatory status[2]. Clinical trials have also addressed the question of a possible

positive impact of antibiotic therapy on the clinical burden from cardiovascular disease[3]. *H pylori* is the most common gastrointestinal agent worldwide. *H pylori* infection has been linked to other non-classical markers of atherosclerotic diseases, such as heat shock proteins[4], and the relationships between infection and these markers have been largely unexplored.

There is now clear evidence that cytotoxic strains of *H pylori* bearing the cytotoxin-associated gene A (CagA) have greater potential for eliciting systemic immune responses. Several subsequent case-control and cohort studies have assessed the association between CagA status and atherosclerotic diseases. Patients with atherothrombotic disease seem more likely to be infected by CagA-bearing strains of *H pylori* than healthy subjects[5]. As a consequence, these studies have assessed a wide variety of *H pylori* CagA-bearing strains, with inconsistent results worldwide, possibly because of the lack of adequate statistical power, selection bias or population diversity. A meta-analysis may avoid some of these methodological difficulties. In this article, we have performed for the first time a meta-analysis of population-based studies, and examined the evidence on the relationship between CagA status of *H pylori* strains and the risk of cerebral infarction (not including cerebral embolism) in European Caucasian and Chinese Han populations.

The aims of the present meta-analysis were: (1) To examine if there is an association between cerebral infarction and more virulent, CagA-bearing strains *H pylori*; (2) to confirm if there is an association between cerebral infarction and CagA-negative strains of *H pylori*; and (3) to confirm if there exists a difference between European Caucasians and Chinese Han.

MATERIALS AND METHODS

Data sources

The present meta-analysis follows the MOOSE statement guidelines[6]. We performed an electronic search of the MEDLINE (1966 to May 2007), Cochrane library (issue 2, 2007) EMBASE (1966 to May 2007), CBMdisc (Chinese Biomedical Literature analysis and retrieval system for compact disc, 1978 to May 2007) and CNKI (China National Knowledge Infrastructure, 1994 to May 2007) databases. In order to reduce publication bias, we collected data by contacting investigators to request unpublished data. Although it was virtually impossible to obtain all unpublished data, we conducted a manual search from the references of the papers retrieved, important academic conference articles and important Masters and Doctoral dissertations. We searched for the following key words from the MeSH index: "*Helicobacter Pylori*", "cytotoxin-associated gene-A", "atherosclerosis", "cerebral infarction", "cerebral ischemia", "stroke", "vascular", "ischemic cerebrovascular disease" and "atherosclerotic cerebral infarction". The languages were restricted to Chinese and English.

Selection criteria

We restricted our review to case-control studies and prospective studies on CagA-bearing strains of *H pylori* and the risk of cerebral infarction; the individuals in case and control groups were all European Caucasians or Chinese Han. We excluded abstracts, review articles, editorials, commentaries and book chapters. Thus, inclusion criteria for the studies were: (1) The demographic characteristics of cases and controls are similar; (2) a confirmed diagnosis of cerebral infarction, and the diagnosis was valid and specified (that is, CT or MRI scan, or a diagnosis of carotid atherosclerosis according to transcranial Doppler ultrasound); (3) cases and controls are similar in terms of all important features except for the disease in question, or they are matched, or adjustment is performed in the calculation of odds ratios; (4) information about exposure was gathered in the same way from cases and controls; (5) validated testing tool(s) for detecting the CagA status of *H pylori* and *H pylori* strains were used; and (6) detection of CagA status was performed blindly.

Studies were excluded when raw data had been duplicately published or the principal investigator was not contacted and couldn't provide the raw data. All identified publications were independently evaluated and selected by two investigators (Zhang S. and Guo Y.) based on their compliance with these criteria. The results were compared and disagreements were resolved by consensus.

Statistical analysis

The aim of this meta-analysis was to assess the relationship between the CagA-bearing strains of *H pylori* and cerebral infarction. To have an internal control, valid in all studies, for this association, valid in all studies, we also assessed the relationship between CagA-negative strains of *H pylori* and cerebral infarction. Because there are no specific tests for detecting CagA-negative strains, for the purposes of the present analysis, we calculated the prevalence of CagA-negative strains of *H pylori* by subtracting the number of CagA-seropositive subjects from the total number of individuals with *H pylori* infection reported in each study.

Data analysis was performed using Review Manager 4.2.10 software (available from the Cochrane Collaboration at http://www.cochrane.org, accessed on June 12, 2007). The studies were pooled by averaging the ORs and 95% CIs with 2-tailed *P* values through a fixed-effects model, using the Mantel-Haenszel approach when no heterogeneity was observed among studies. When evidence of heterogeneity among studies was detected, a random-effects model was adopted for a combination of all studies. Heterogeneity among studies was tested by the Chi-square statistic, obtained by summing up the weighted squares of the deviations of each estimate from the pooled estimate ($P < 0.05$ was considered evidence of heterogeneity).

Lack of adjustment for potential confounders and classic cardiovascular risk factors may inflate estimates of any association of novel candidate risk factors with atherosclerotic cerebral infarction. We performed a sensitivity analysis, with the same statistical methods used for the main analysis, excluding studies that did not provide an adjusted odds ratio.

Publication bias was examined by plotting a funnel plot of the reported effect and fail-safe number (Nfs) methods[7]. We plotted the studies' log odds ratio versus the sample sizes to detect asymmetry in the distribution

Table 1 Characteristics of the studies included in the meta-analysis

Author	CagA-bearing strains		CagA-negative strains		Detection method	Race	Multivariate adjustment	Publication year
	In case	In control	In case	In control				
Li[9]	38/83	14/71	34/83	26/71	ELISA	Chinese Han	Age, sex, main risk factors[1], cardiogenic factors, occupation	2001
Wang[8]	28/79	11/73	32/79	28/73	ELISA	Chinese Han	Age, sex, main risk factors[1], cardiogenic factors, hypercoagulable states	2004
Liu[10]	21/50	19/50	15/50	18/50	ELISA	Chinese Han	Age, sex, main risk factors[1], occupation	2003
Sun[12]	47/102	13/72	43/102	18/72	ELISA	Chinese Han	Age, sex, main risk factors[1], cardiogenic factors, family history, education	2006
Wu[13]	41/78	21/60	19/78	25/60	ELISA	Chinese Han	Age, sex, main risk factors[1], drinking	2003
Wang[11]	62/100	19/50	9/100	15/50	ELISA	Chinese Han	Age, sex, main risk	2006
Preusch[14]	34/92	45/229	34/92	82/229	ELISA	German Caucasian	Age, sex, main risk factors, occupation, family and past history, cardiogenic factors, social class	2004
Gabrielli[15]	44/105	30/130	31/105	51/130	ELISA	Italian Caucasian	Age, sex, main risk factors[1], BMI, family history, occupation, peptic ulcer,	2004
Pietroiusti[16]	59/138	27/151	36/138	79/151	ELISA	Italian Caucasian	Age, sex, main risk factors[1], BMI, social class, peptic diseases, occupation	2002
Majka[17]	46/80	27/80	23/80	27/80	ELISA	Polish Caucasian	Age, sex, diabetes, hypertension, social class	2002

[1]Main risk factors: Cigarette smoking, hypertension, diabetes, hyperlipidemia.

of trials. A gap in the funnel plot indicates potential for publication bias.

RESULTS

Study retrieval and inclusion

We found a total of 10 retrospective case-control studies on CagA-bearing strains of *H pylori* and cerebral infarction that were eligible for inclusion; these included 6 studies[8-13] of Chinese Han and 4 studies[14-17] of European Caucasians. We performed a meta-analysis of these studies, dividing the overall population into Chinese Han and European Caucasian subgroups, yielding a total of 907 "cases" and 966 "controls". The study parameters are summarized in Table 1.

All of the included studies adjusted data for important confounding factors, the important confounding factors (for example, age, gender, diabetes, hypertension, smoking, social class, BMI, hyperlipidemia, peptic diseases and other pathogenic micro-organism infection) between groups were comparable, and 6 studies[8,11,12,14-16] performed multivariate analysis. All studies used enzyme-linked immunosorbent assay (ELISA) to detect seropositivity for the CagA antigen.

Result of meta-analysis on the relationship between CagA-bearing strains of H pylori and cerebral infarction

The results of tests for heterogeneity in the overall population and its subgroups revealed homogeneity in these studies ($P = 0.29$ in Chinese Han; $P = 0.77$ in European Caucasian and $P = 0.61$ in overall population); therefore, we adopted a fixed effect model to perform combined analysis. The results of combined analysis were statistically significant (OR = 2.60, 95% CI: 1.93-3.49, $P < 0.00001$ in Chinese Han; OR = 2.71, 95% CI: 2.05-3.59, $P < 0.00001$ in European Caucasians; OR = 2.66, 95% CI: 2.17-3.26, $P < 0.00001$ in the overall population), suggesting CagA-bearing strains may be a determinate predisposing factor for the risk of cerebral infarction in the overall population. Moreover, it is also suggested there is no significant difference between Chinese Han and European Caucasians. The results of meta-analysis are shown in Figure 1.

Result of meta-analysis on the relationship between CagA-negative strains of H pylori and cerebral infarction

The test for heterogeneity suggested there was significant in these studies ($P = 0.001$ in Chinese Han; $P = 0.009$ in European Caucasian and $P < 0.0001$ in overall population). We performed combined analysis with a random effect model, yielding ORs of 0.81 (95% CI: 0.45-1.48, $P = 0.50$) in Chinese Han; 0.64 (95% CI: 0.37-1.09, $P = 0.10$) in European Caucasians and 0.74 (95% CI: 0.49-1.10, $P = 0.14$) in the overall population, indicating there was no apparent association between CagA-negative strains of *H pylori* and cerebral infarction. The results were similar in both the Chinese Han and European Caucasian cohorts. The results of meta-analysis are shown in Figure 2.

Publication bias

The publication bias of the meta-analysis on the relationship between the CagA status of *H pylori* strains and cerebral infarction was detected by the Funnel Plot and fail-safe number methods. Although no major asymmetries were detectable in the plot (see Figures 3 and 4), the small number of studies retrieved did not allow publication bias to be excluded. Applying fail-safe number methods (with $P = 0.05$ and $P = 0.01$) to evaluate publication bias suggested there was no potential for lack of published data for the negative outcomes. (Nfs0.05 = 81.70, Nfs0.01 = 37.45 in Chinese Han; Nfs0.05 = 169.96, Nfs0.01 = 82.18 in European Caucasians and Nfs0.05 = 498.68, Nfs0.01 = 242.01 in the overall population).

Sensitivity analysis

All of the studies retrieved considered important and

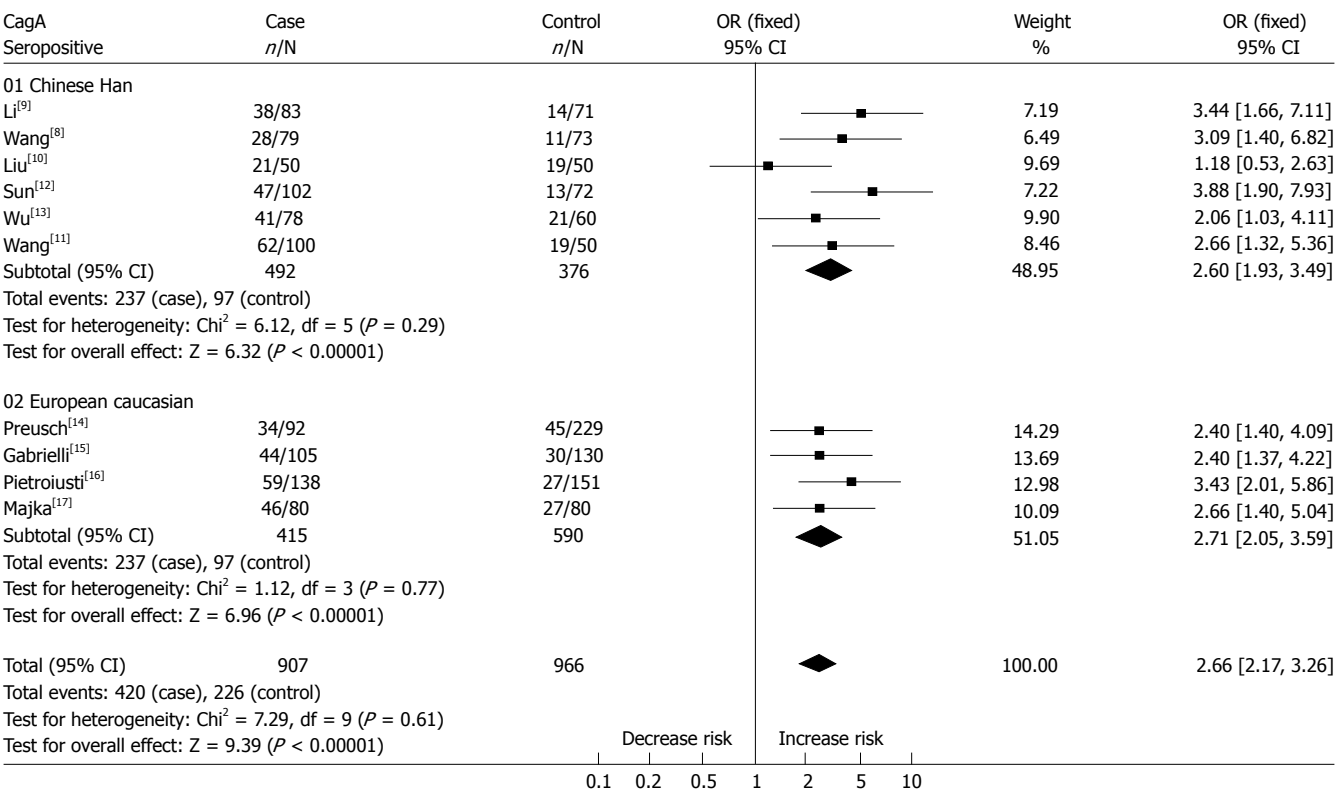


Figure 1 Relationship between CagA-seropositive strains and cerebral infarction.

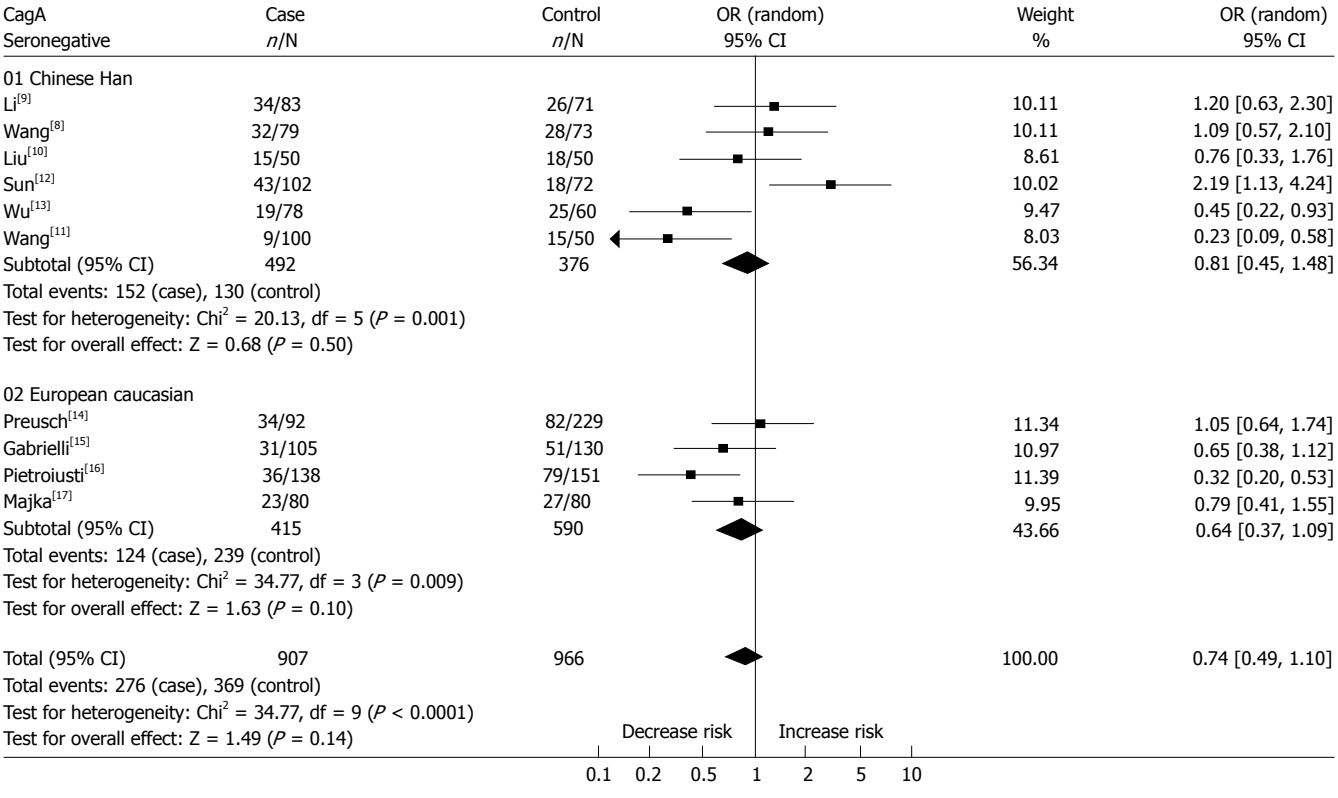


Figure 2 Relationship between CagA-seronegative strains and cerebral infarction.

potential confounders and classical cardiovascular disease risk factors. However, there were four studies, three[9,10,13] in the Chinese Han subgroup and one[17] in the European Caucasian subgroup that did not provide adjusted odds ratios for the relationship between cerebral infarction and CagA-bearing strains. The sensitivity analysis performed after excluding studies without adjusted odds ratios gave a pooled odds ratio of 2.89 (95% CI: 2.25, 3.71, $P < 0.0001$)

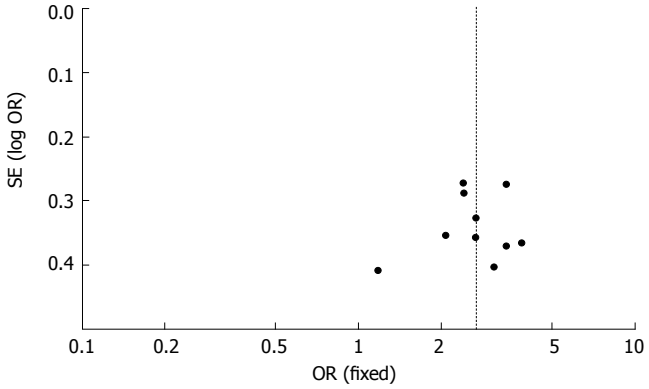


Figure 3 Funnel plot for CagA-seropositive strains.

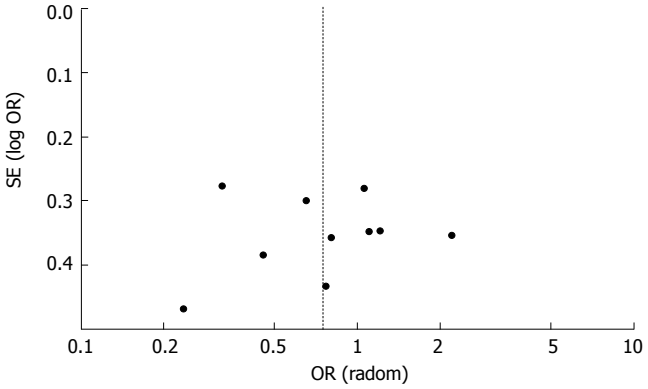


Figure 4 Funnel plot for CagA-seronegative strains.

in the overall population. These results were similar to the results of the meta-analysis before the sensitivity analysis, suggesting the combined results of meta-analysis were reliable.

DISCUSSION

Infection by *H pylori* is one of the most common chronic infections, occurring in approximately half of the world's population. It commonly affects the gastric mucosa[18,19]. Recent studies have found evidence that has resulted in *H pylori* strains being approximately classified into three types; type I includes highly virulent strains expressing CagA toxin and VacA toxin, which is strongly related to CagA. Recent studies have found evidence of *H pylori*, especially CagA-bearing strains, in atherosclerotic plaques[20,21] and an association with atherosclerotic diseases.

Using meta-analysis, we found a significant association between cerebral infarction, particularly that caused by atherothrombosis of large vessels, and CagA-bearing strains of *H pylori*, but not CagA-negative strains. In addition, no difference was found between Chinese Han and European Caucasian populations. By applying sensitivity analysis, we excluded articles that did not provide an adjusted odds ratio, and no difference was found in the conclusion. This indicated the conclusion was reliable and credible. Conducting chronic infected by CagA seronegative strain as the risk factor to combine analysis, we found no statistical significance between CagA-negative strains of *H pylori* and cerebral infarction in both subgroups and the overall population. Thus, it was uncertain whether CagA-negative strains of *H pylori* increase the risk of cerebral infarction.

Several mechanistic models may be invoked to explain the association we identified in our meta-analysis. First, it is well known CagA-bearing strains are able to induce enhanced inflammation in the gastric mucosa[22]. Since inflammation plays a major role in atherosclerosis and complicated lesions are associated with an increased inflammatory response[23], the strong inflammatory response related to infection by CagA-bearing strains may influence process of atherosclerosis through an immune-mediated release of cytokines and other substances endowed with pro-inflammatory properties[16,17]. Second, it has been suggested *H pylori*, as well as Chlamydia pneumoniae, could directly provoke inflammation within atherosclerotic plaques. *H pylori* DNA has been found in carotid atherosclerotic lesions and is associated with features of inflammatory responses[20]. Third, an auto-immune reaction could be postulated. Cross-reactivity between anti-CagA antibodies and vascular wall antigens has been recently demonstrated, suggesting these antibodies may contribute to the activation of inflammatory cells within atherosclerotic lesions[24]. This activation might eventually lead to plaque destabilization.

Although the results of the meta-analysis were positive, it is important to note there were no prospective studies on CagA status and risk of ischemic cerebral diseases. The prospective data available on the topic come from four studies that gave contrasting results on the association between *H pylori* infection and stroke[25-28]. Regrettably, these studies were heterogeneous in design and provided no raw data to calculate a pooled odds ratio. Only one of these studies[25] reported a large prospective study that compared CagA-seropositive and *H pylori*-seronegative subjects with regard to the relationship between CagA status and progression of carotid atherosclerosis, during a 5-year follow-up; and the authors showed that infection by CagA-bearing strains of Helicobacter is associated with a larger increase in carotid intimal-medial thickness (IMT) and concluded that infection with CagA-bearing strains could contribute to the pathogenesis of early atherosclerosis. However, no data are available for clinical end points. One study[28] also found that CagA-positive atherosclerotic stroke patients had poorer initial outcomes based on serial measurements of NIHSS scores. No significant differences in IMTs and NIHSS scores were found between *H pylori*-positive and *H pylori*-negative patients. Ischemic cerebrovascular diseases, especially cerebral infarction, is likely to be multifactorial[29], involving both polygenic and environmental influences. This article has provided evidence of an association between CagA status and the risk of cerebral infarction in Chinese Han and European Caucasians. It is important to bear in mind the observation nature of meta-analysis, so the standard of quality control could not be completely identical[30]. However, the majority of studies included performed multivariate adjustment in order to minimize bias. Moreover, the sensitive analysis, funnel plot and fail-

safe number analysis performed in this article could avoid and evaluate the bias to some extent. More efforts are still needed to perform an accumulated meta-analysis by conducting a high-quality prospective study with expanded sample size, so as to lay a solid foundation for further combined research. Prospective evidence will also clarify the impact of *H pylori* eradication on the risk of cerebral infarction, and may clarify the relationship between them.

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COMMENTS

Background

Accumulated evidence indicates a variety of infections contribute to the pathogenesis of atherosclerosis and cerebral infarction, but conflicting results have emerged from the literature. To assess the association between the cytotoxin-associated gene-A (CagA) status of *H pylori* strains and the risk of cerebral infarction, we carried out a meta-analysis of all related studies.

Research frontiers

There have been many clinical and experimental observations highlighting the important role of chronic CagA-bearing strains of *H pylori* in the development of cerebral infarction. Our study provides evidence for the possible involvement of CagA-bearing strains in cerebral infarction by enhancing some risk factors of atherogenesis. Moreover, the investigation into two genetically distinct populations is of interest.

Innovations and breakthroughs

The most interesting result of this study is we have performed a systematic meta-analysis of population-based studies investigating CagA-bearing and CagA-negative strains of *H pylori* and their association with the risk of cerebral infarction. The association between *H pylori* and acute cerebrovascular disease seems to be due to a higher prevalence of more virulent *H pylori* strains in patients with atherosclerotic stroke.

Applications

The meta-analysis suggests *H pylori* is associated with other markers of atherosclerosis ischemic stroke. The presence of CagA-bearing strains of *H pylori* causing chronic inflammation is a statistically significant risk factor for cerebral infarction. this article is valuable for the prevention and prediction of cerebral infarction in high-risk populations.

Terminology

Meta-analysis is a statistical tool for combining results across studies; it is becoming popular as a method for resolving discrepancies in association studies of risk factors.

Peer review

This is a well-conducted meta-analysis.

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CASE REPORT

A rare presentation of appendiceal diverticulitis associated with pelvic pseudocyst

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Abstract

We report a case of a 32-year-old woman with episodic right lower quadrant abdominal pain. With the diagnosis of either acute appendicitis or tubo-ovarian abscess, she was treated with antibiotics, which successfully relieved the pain and the inflammatory findings. She repeated the clinical condition a few times and antibiotics worked each time. In a year, her subjective symptoms became milder, however, a giant pelvic cyst appeared. She had an exploratory laparotomy to confirm this diagnosis. Histopathological studies revealed herniated appendiceal mucosa through the muscular layer associated with chronic inflammation and marked fibrosis. Gynecological disorders such as endometriosis or lutein cyst rupture was denied. These findings represent appendiceal diverticulitis. We discuss the clinical features of the disease and its relation with the pelvic pseudocyst.

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Key words: Appendiceal diverticulitis; Perforation; Pelvic pseudocyst

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CASE REPORT

A 32-year-old woman visited our hospital with diarrhea, abdominal pain and fever. With the diagnosis of acute enteritis, an oral antibiotic was given, which resolved her symptoms. Eight months later, pain in the upper abdomen, then in the lower, appeared. Inflammatory markers such as WBC and CRP arose up to 13.0 × 10^9/L and 57 mg/L respectively. Ultrasonography detected no more than the wall thickening of the small intestine. Enteritis was the diagnosis and antibiotics worked again. Two months later, upper stomachache relapsed, which afterwards moved to the right lower quadrant. At this time, the pain was accompanied by remittent fever up to 39 centigrade. Pelvic CT scan showed a whip-shaped low-density structure with a high-density spot at its caudal end extending downwards along the bulging right pelvic wall which was then assumed to be the effect of inflammation (Figure 1). The lesion was thought to be either the swollen appendix with a fecal stone or tubo-ovarian abscess with calcification. In order to be effective in either case, an intravenous antibiotics, flomoxef sodium, was infused for a week, which successfully subsided the inflammatory symptoms but occasional abdominal pain. In four months, lower abdominal pain, neither accompanied by high fever nor related with gynecological episodes such as ovulation or menstruation, gradually became worse. The laboratory data suggested the presence of inflammation again, with WBC 11.7 × 10^9/L and CRP 45 mg/L. The outstanding finding of CT scan was a cyst in cyst structure in the pelvis, with the diameter of 3 cm and 8 cm respectively. The high density spot, without low density lesion nearby at this time, relocated cranially into the right iliac fossa and the bulging of the right pelvis disappeared (Figure 2A and B).

Though our initial diagnosis was the pelvic abscess caused by appendiceal perforation, the abscess of tubo-ovarian origin was still possible. So, laparotomy was chosen to confirm the diagnosis and the treatment.

At laparotomy, peritoneal cavity was free of ascites. The terminal ileum and the top of the uterus adhered to each other to block the exploration down the pelvic cavity. While dissecting the conglomerated organs, the dark brown serous fluid without pus-like odor or feel came out. Then, the ruptured cyst with the right ovary appeared. The atrophic appendix adhered to the back of the cecum, being entirely wrapped by dense fibrous tissue.

Though other cysts were not elsewhere as far as we explored, we tentatively made the diagnosis of ovarian endometriosis. That was because the inner wall of the cystic lesion was chocolate brown and bumpy, and in addition, the appendix lacked acute inflammatory findings. We first resected the right ovary and tube together and scraped the ruptured cystic wall as much as possible. Then appendectomy was added. She recovered well except the

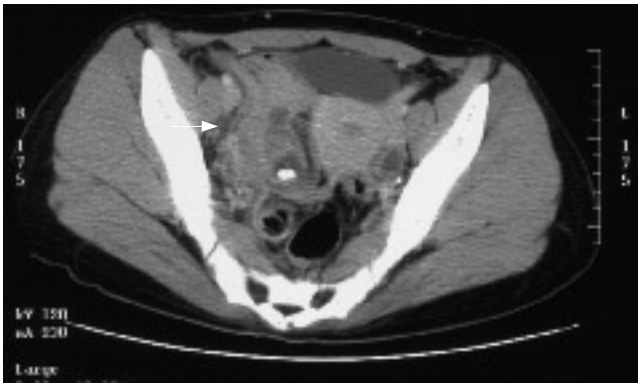


Figure 1 Pelvic CT shows a whip-shaped low density structure with a high density spot along the bulging right pelvic wall.

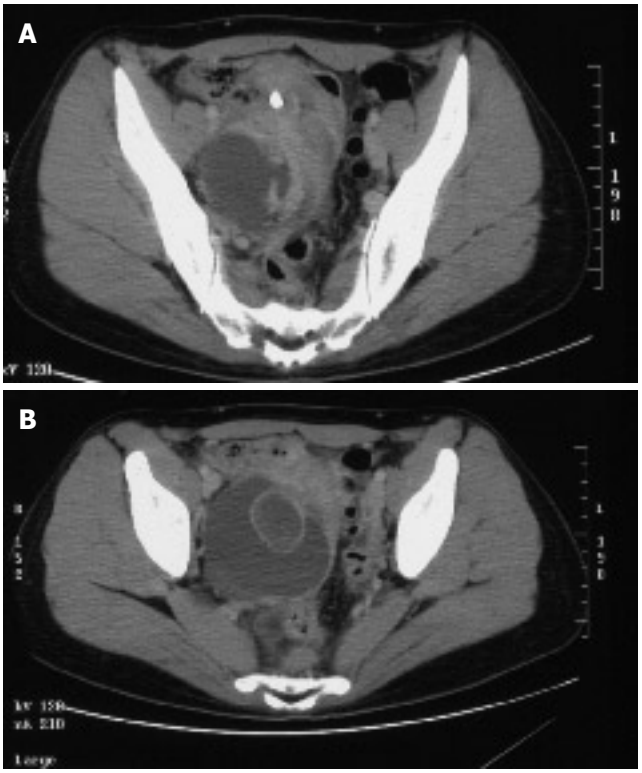


Figure 2 Pelvic CT in 4 mo. A cyst in cyst structure and the high density spot in the right iliac fossa were revealed.

wound infection at the subdermal level and was discharged on the postoperative d 14. The abdominal annoyances have not recurred since then.

The pathologic examination was as follows. The cystic wall did not have any endometrial components, but had hemosiderin deposits, which suggests a pseudocyst with old bleeding. The ovarian cyst was composed of luteal body and follicles. The appendix had a mucosal protrusion in the subserosal layer near its orifice, which is thought to be a diverticulum. Only chronic inflammation was found both along the peridiverticulum and in the whole appendix.

DISCUSSION

Appendiceal diverticulum is classified as either congenital or acquired form like other intestinal diverticulum[1]. The congenital diverticulum has full layers, that is, mucosa, submucosa, muscle, and serosa, and therefore it hardly perforates. The congenital form is exceedingly rare and was found in 0.0014% in the study of a series of 50 000 appendices[2]. The acquired form lacks muscular layer, which explains its mechanical weakness and inclination to perforation[1]. The acquired one is more common and its incidence ranges from 0.004% to 2.1%[1]. Diverticula are usually located in the distal one third of the appendix[2]. A high proportion of diverticula occurs along the mesenteric border, which is structurally weak because of the existence of vascular hiatuses[3]. Although appendiceal diverticulitis presents with similar signs and symptoms to appendicitis, its clinical features are different in some ways. Appendiceal diverticulitis is seen mainly after the third decade of life while the average age of appendicitis patient is 19 years[1,4,5]. The disease is more common in men, is usually accompanied by previous attacks of pain, and presents fewer gastrointestinal symptoms[1,4].

Those with appendiceal diverticulitis are four times more likely to have a perforation compared with simple appendicitis, which is thought to be responsible for a longer convalescence and 30-fold more mortality[1,4,6,7]. Importantly, the incidence of perforation in cases of diverticulitis was 66%[6]. However, the way of perforation, whether to free peritoneal cavity or into retroperitoneal space is not well documented. Simpson *et al* reported a case of diverticular abscess of the appendix in the right iliac fossa[4]. According to their CT figures, the abscess extends over to the preperitoneal space anteromedial to the right iliac crest, which implies retroperitoneal perforation of the diverticulum. Place *et al* reported a case of chronic appendicitis with diverticular formation of which CT finding suggested extensive inflammation in the right retroperitoneum and pericecal area[1].

Since most acquired appendiceal diverticula are on the mesoappendix, the inflammatory process may well be contained within the mesentery after perforation and cellulitis may develop in the nearby retroperitoneum. That may be one of the reasons why generalized peritonitis is uncommon in the appendiceal diverticulitis[7].

In acute appendicitis, on the contrary, perforation usually heads for the free peritoneal cavity followed by diffuse peritonitis.

In our case, we speculate that the appendiceal diverticulum also perforated into the retroperitoneal space and made phlegmon beneath the right pelvic peritoneum. Considering the above-mentioned case reports, we assume that inflamed diverticula may be apt to perforate into a less symptom presenting space, that is, retroperitoneum, at an earlier inflammatory stage. Moreover, unless appendicitis is accompanied, significant findings are hard to get by imaging tests. Prescribing antibiotics without keeping in mind the possibility of diverticulitis might just complicate the symptoms and make it insidious. Complication of a large pelvic cyst with appendiceal diverticulitis like our case has not been reported as far as we searched. Since the large cyst was not present 4 mo before and gynecological disorder such as endometriosis is pathologically unlikely, it should be related to diverticulitis. It is possible that

the permeated appendiceal fluid brought about cellulites underneath the right pelvic retroperitoneum, which eventually developed the pooling of infectious exudates. We speculate that during the course of treatment, it may have re-perforated into the free pelvic cavity to form a large pseudocyst, while appendiceal diverticulitis became chronic and almost healed. The treatment strategy of appendiceal diverticulitis should depend on its degree and spread of inflammation. Because its direct diagnosis is not easy, in the patients with repeating lower abdominal pain with inflammatory findings, we should take this clinical entity into account.

We experienced a case of appendiceal diverticulitis associated with a large pelvic pseudocyst. We speculate that the appendiceal diverticulum perforated into the retroperitoneal space and made phlegmon beneath the right pelvic peritoneum, which eventually developed the pooling of infectious exudates. Then, during the course of treatment, it may have re-perforated into the free pelvic cavity to form a large pseudocyst. Since appendiceal diverticulitis has 30 times mortality compared with simple appendicitis, to recognize this disease is clinically most important.

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CASE REPORT

A squamous metaplasia in a gastric ulcer scar of the antrum

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Abstract

An 81-year-old man presented with epigastric pain and weight loss for one month. He had a past history of pulmonary tuberculosis, 10 years ago. We performed a gastroscopy, which showed a linear depressed whitish gastric ulcer scar (0.8 cm in length) in the posterior wall of the prepyloric antrum. The result of biopsy was reported as squamous epithelium. Immunohistochemical staining using an antibody to high molecular weight cytokeratin (HMC) revealed positive staining in the squamous epithelium. Two years later, the lesion was followed up. The lesion remained at same site endoscopically, but no squamous epithelium could be seen microscopically.

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Key words: Squamous metaplasia; Gastric ulcer scar; High molecular weight cytokeratin

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INTRODUCTION

Squamous metaplasia of the stomach is not a rare clinical entity associated with gastroesophageal reflux disease and Barrett's esophagus at the cardia of the stomach[1-3]. It has been rarely described with other associated diseases, such as peptic ulcers[4], tuberculosis[5], syphilis[6], corrosive gastritis[7], extreme cachexia[8], pernicious anemia[9] and aberrant pancreatic tissue[10]. It may coexist with neoplasms[11,12].

However, there has been no report of squamous metaplasia occurring at an old ulcer scar site in the distal antrum with none of the associated diseases described above. We confirmed squamous metaplasia by a forcep biopsy with immunohistochemistry; it had disappeared spontaneously, microscopically, by the time of follow-up gastroscopy biopsy two years later.

CASE REPORT

The patient was an 81-year-old Korean man who was admitted to the authors' institution because of general weakness and loss of weight of 1 mo duration. He had pulmonary tuberculosis 10 years before admission. The patient was found to be thin, weighing only 36 kg. Breathing sound was decreased on the left lung field. Physical examination revealed no abnormal findings in the abdomen or elsewhere. The hematology values were more or less normal. Stool was negative for occult blood. Urinalysis and ECG were normal. Endoscopy revealed a linear depressed whitish old gastric ulcer scar of about 0.8 cm in length in the posterior wall of the prepyloric antrum (Figure 1). The lesion was slightly depressed. There was no evidence of malignancy in the biopsy, and squamous epithelium was obtained from the area (Figure 2). Immunohistochemical staining using an antibody against high molecular weight cytokeratin (HMC) (Moll's No 1, 5, 10, 14) from Dako (Carpinteria, California, USA) revealed positive staining in the squamous epithelium (Figure 3). Two years later, follow-up gastroscopy was performed, and the lesion was still present (Figure 4). A biopsy showed no evidence of malignancy, and no squamous epithelium was found. The patient is continuing to receive follow-up examinations in our hospital.

DISCUSSION

Squamous cell metaplasia of the stomach is not rarely reported in association with gastroesophageal reflux and distal esophageal inflammation. Takeda *et al* described

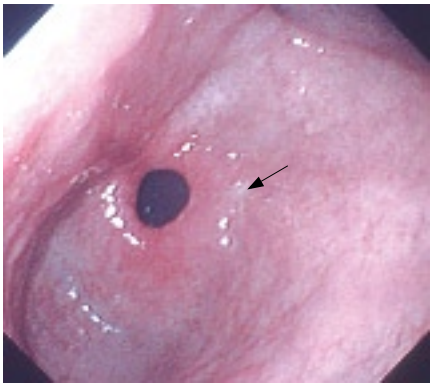


Figure 1 EGD showing a linear depressed whitish mucosal lesion of about 0.8 cm in length (arrow).

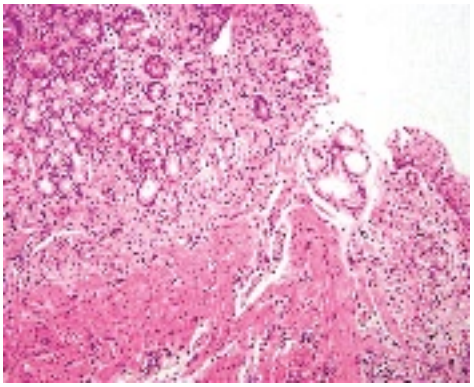


Figure 2 Photomicrograph showing squamous metaplasia (HE; original magnification, × 100).

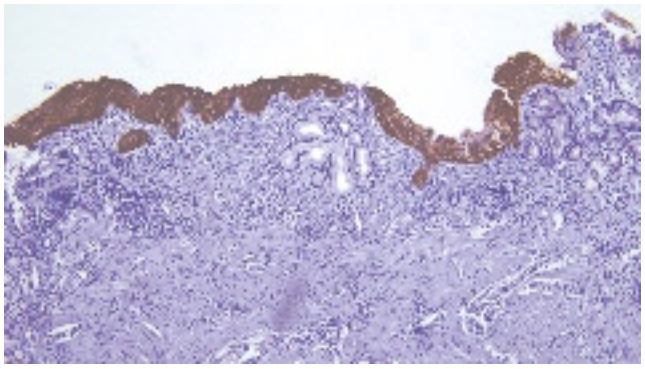


Figure 3 Photomicrograph showing an immunohistochemical demonstration of high molecular weight cytokeratin (original magnification, × 40).



Figure 4 Follow-up EGD showing a linear depressed whitish mucosal lesion without interval change (arrow).

two cases of squamous metaplasia in the lesser curvature of the cardiac region, which were recognized as white or pale mucosal areas, and the lesions were slightly depressed when sprayed with indigo carmine[1]. It has been known squamous cell metaplasia may affect the lesser curvature of the stomach preferentially, if it does not spread into the entire stomach[4]. In our case, squamous metaplasia developed at an old gastric ulcer scar site in the prepyloric antrum; this is the first reported case of squamous metaplasia in such a location and without associated disease. We confirmed the squamous metaplasia by forcep biopsy combined with immunohistochemistry (for HMC). To our knowledge, the present case is the first to describe positive immunohistochemical staining using an antibody against HMC. The distinctive staining pattern by HMC antibodies may help in the identification of squamous metaplasia. In animal studies, gastric squamous metaplasia has been experimentally induced in by the injection of pyrogallic acid[13] and methylcholanthrene[14].

It has been postulated the squamous epithelial cells develop as a progeny of a multipotent stem cells[15]. However, the pathogenesis of squamous metaplasia in the stomach remains speculative. Also, in these cases, the underlying pathogenesis of metaplasia is unknown, but it is thought to occur transiently, induced by gastric stem cells during the healing of gastric ulcers. This transient phenomenon of metaplasia was confirmed by comparing the results of the initial biopsy with those of a follow-up biopsy performed two years later. Whether squamous metaplasia of the stomach represents a premalignant change is not known. However, some reports have shown squamous cell carcinoma of the stomach sometimes develops from squamous metaplasia[4,6,7,11]. It is thought this case could have developed from gastric stem cells ontogenically, like a gastric squamous cell carcinoma. In our case, no tumorous change occurred at the metaplasia site during a two year follow-up period. Based on this evidence, close endoscopic follow-up is necessary for patients with squamous metaplasia in whom the pathogenesis of metaplasia remains to be elucidated.

In conclusion, we report a case of squamous metaplasia on the posterior wall side of the prepyloric antrum of the stomach, in an elderly male patient. Squamous metaplasia should be considered in patients with a white or pale depressed mucosal area by endoscopic examination. Careful and close endoscopic follow-up is warranted for patients with squamous metaplasia.

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CASE REPORT

Carcinoid tumor of the common bile duct: A rare complication of von Hippel-Lindau syndrome

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Abstract

Von Hippel-Lindau syndrome (VHL) is a rare autosomal-dominant, inherited familial cancer syndrome. Hemangioblastomas, pheochromocytomas and renal carcinoma are the frequent reported VHL tumors. Neuroendocrine tumors have also been described, mostly in the pancreas and rarely in the biliary trees. We report the second case of bile duct carcinoid in a 31-year-old VHL woman. She underwent right adrenalectomy for a pheochromocytoma in the past. She also had a positive family history of phenotypic expression of VHL syndrome. The patient presented with biliary colic. Endoscopic retrograde cholangio-pancreatography showed intra-luminal bile duct mass. Surgical exploration identified a beige nodular lesion that was a carcinoid tumor on histology. This new association should be clarified by further genetic investigations.

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Key words: Carcinoid tumor; Bile duct tumor; Von Hippel-Lindau syndrome

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INTRODUCTION

Von Hippel-Lindau syndrome (VHL) syndrome is a rare autosomal-dominant, inherited familial cancer syndrome. It is caused by germline alterations in the VHL tumor suppressor gene (3p25)[1]. It has a reported incidence of 1/36 000 births. Twenty percent of patients have no familial history[1]. This germline mutation is associated with a significant variability in the expression of the different tumor associated with the syndrome[1]. The VHL gene codes a protein whose physiological function is the down-regulation of transcription activity of cell growth and cell cycle[2]. VHL gene inactivation may contribute to VHL tumorigenesis[2]. Most frequent VHL tumors include hemangioblastomas of the central nervous system and/or retina, pheochromocytomas (often bilateral), renal carcinoma and pancreatic cystic tumors[1]. Neuroendocrine tumors (NETs) have been reported in VHL patients, mostly of the pancreas[3]. In the bile duct, only one case of NET has been described in VHL patients[4]; we report the third case over the last 16 years.

CASE REPORT

A 31-year-old woman was routinely screened for von Hippel-Lindau (VHL) syndrome since 1998 after undergoing partial right adrenalectomy for a pheochromocytoma. She had a positive family history of phenotypic expression of VHL syndrome. Her mother was asymptomatic, but her brother was submitted to bilateral adrenalectomy for pheochromocytoma. In 2005, she underwent right adrenalectomy for recurrent pheochromocytoma. A few months later, she also underwent cholecystectomy for symptomatic cholelithiasis.

Six months post cholecystectomy, she presented with biliary colic. Abdominal ultrasound showed normal liver architecture and non dilated bile ducts. Endoscopic retrograde cholangio-pancreatography (ERCP) showed a distal partial filling defect compatible with an intra-luminal bile duct mass (Figure 1). No evidence of recurrence of pheochromocytoma was identified on abdominal CT scan.

The patient was then referred for complementary management. At the time of assessment, she was totally asymptomatic. Her weight was stable. Physical examination was unremarkable. The liver function tests as well as the CA19-9 level were completely normal. Urinary catecholamine level was normal. Endoscopic ultrasound revealed an intra-luminal bile duct polypoid solid mass of

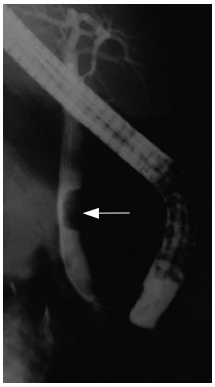


Figure 1 ERCP showing intra-luminal bile duct lesion.

16 mm in diameter. Fine needle aspiration biopsy as well as brush cytology were negative twice.

Based on these findings, surgical exploration was considered. At laparotomy, an intra-luminal bile duct lesion was palpated. Bile duct resection, hilar node dissection and hepatico-jejunostomy bile duct reconstruction was performed. The post operative course was uneventful. Macroscopically, the lesion was a beige nodular lesion measuring 1.2 cm × 1 cm. Pathological analysis reported a well-differentiated carcinoid tumour of the common bile duct. It is considered to be a complication of VHL syndrome (Figure 2).

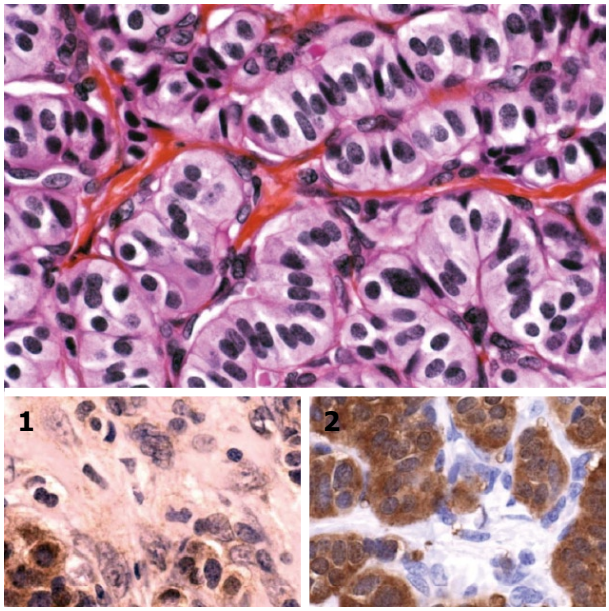


Figure 2 Trabecular pattern of neuroendocrine cell proliferation (HPS, × 200). Insert: (1) Strong staining of all tumor cells (NSE, × 200). (2) S-100 nuclear staining of 20% of tumor cells which also co-expressed NSE (× 400). No sustentacular staining pattern. NSE: neuron-specific enolase; HPS: Hematoxyline-phloxine-safran.

DISCUSSION

VHL syndrome is a rare autosomal-dominant, inherited familial cancer syndrome. It is caused by germline alterations in the VHL tumor suppressor gene (3p25). It has a reported incidence of 1/36 000 births. Twenty percent of patients have no familial history[1]. This germline mutation is associated with a significant variability in the expression of the different tumors associated with the syndrome[1]. The VHL gene codes a protein whose physiological function is the down-regulation of transcription activity of cell growth and cell cycle[2]. VHL gene inactivation may contribute to VHL tumorigenesis[2]. Most frequent VHL tumors include hemangioblastomas of the central nervous system and/or retina, pheochromocytomas (often bilateral), renal carcinoma and pancreatic cystic tumors[1]. The lifetime risk for developing these tumors is about 70%. It varies according to the nature of the germline mutation[1]. Globally, tumors in VHL syndrome occur at an earlier age than usual and are often multifocal[2]. Neuroendocrine tumors (NETs) have been reported in VHL patients, mostly of the pancreas[3]. NETs are derived embryologically from enterochromaffin cells. Hammel found hormonally non-functional pancreatic NETs in 12.3% of VHL patients[3]. Lubensky associated NETs with VHL syndrome on the basis of an allelic deletion of the VHL gene[5]. NETs in the bile duct and gallbladder have been described in only two patients with VHL syndrome[4,6]. The rarity of enterochromaffin cells in the biliary tree could explain this observation. Their number increases with intestinal metaplasia of the biliary mucosa consequent to chronic inflammation[7]. In VHL syndrome, the biliary complications are usually associated with pancreatic cystic lesions[8].

Only 49 cases of extrahepatic biliary carcinoid have been reported so far. These tumors occurred mostly in the common bile duct, were non-functional in the majority of cases and affected mostly women in their fifth decade of life. Jaundice was the predominant symptom. Less than one-third of patients present with metastases. Most of them are suitable for curative resection with prolonged disease-free survival[9-11].

Biliary NETs in VHL patients could be an independent tumor type of the syndrome or may only be related by chance. This relationship has been clearly established by genetic investigations for pancreatic NETs. Therefore, the next step in the determination of VHL biliary NET pathogenesis should be the identification of genetic changes at this level.

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CASE REPORT

Gastrointestinal stromal tumour of the rectum: Report of a case and review of literature

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Abstract

Gastrointestinal stromal tumour (GIST) is a rare tumour of the gastrointestinal tract which does not generally originate in the rectum. The authors describe a case of a 70-year-old man who underwent an anterior resection of the rectum for a low-risk GIST. The patient was not given adjuvant chemotherapy with imatinib and is still disease-free 30 mo after surgery. The authors conclude that although rectal GIST is extremely uncommon, it should be included in differential diagnosis when a tumour in the rectum is detected. Biopsy of the tumour is essential, since this makes it possible to reach a sure preoperative diagnosis based on the immunohistological features of the CD117 and CD34. Although complete surgical resection with negative tumour margins is the principal curative procedure for primary and non-metastatic tumours, further studies are still needed for the determination of the most effective treatment strategy for patients with rectal GIST.

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Key words: Rectal; Gastrointestinal stromal tumour; Diagnosis; Treatment; Surgery

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INTRODUCTION

Gastrointestinal stromal tumour (GIST) is a rare tumour involving the gastrointestinal tract and expresses CD117, a c-kit proto-oncogene, which can be detected immunohistochemically[1,2]. The most common GIST sites are the stomach (60%-70%) followed by the small intestine (20%-25%), whereas only about 5% of all GISTs start in the rectum[3,4]. Rectal GISTs make up 0.1% of all tumours originating in the rectum. According to a recent epidemiological investigation conducted in the USA, their incidence is 6.8 per million people annually[3,5]. In this report, the authors describe a rare case of GIST of the rectum.

CASE REPORT

A 70-year-old man was referred to our hospital complaining of a dragging sensation in the rectum and several episodes of rectal pain, without any problems of the alvus. A clinical examination did not detect any palpable abdominal mass. Digital investigation of the rectum revealed a mass of approximately 4 cm in diameter on the left lateral rectal wall at about 2 cm above the dentate line. The mass was hard, elastic and immobile, with an irregular surface. No blood was found on the exploratory finger. A provisional diagnosis of rectal carcinoma was made and a colonoscopy was programmed. This examination confirmed the presence of a mass, probably of submucous origin, measuring about 4 cm in its maximum diameter, attached to the lateral left rectal wall and at 2 cm above the dentate line. The surrounding mucosa proved to be healthy. Endoscopic observation suggested that the lesion probably originated in the connective tissue, transrectal ultrasound was therefore performed, together with an echo-guided needle-biopsy for the histological examination and a computed tomography (CT) with contrast medium of the abdomen and the chest and an assay of the carcinoembryonic antigen (CEA) and Ca19.9 tumoral markers.

Laboratory investigations revealed microcytic hypochromic anaemia, with haemoglobin of 11.9 g/dL, while CEA and Ca19.9 values were normal.

Transrectal ultrasound confirmed a predominantly exophytic, heterogenous, hypoechoic submucosal mass (measuring 35 mm × 26 mm) on the lateral left rectal wall (Figure 1). The histological examination of the biopsy samples led to a diagnosis of GIST due to the

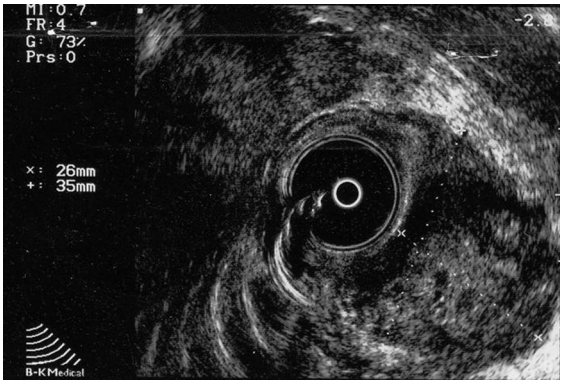


Figure 1 Transrectal ultrasound confirming a predominantly exophytic, heterogenous, hypoechoic submucosal mass (measuring 35 mm × 26 mm) on the lateral left rectal wall.



Figure 2 TC confirming the sonographic findings of the presence of a mass with a marked, irregular, eccentric thickening of the lateral left wall of the lower third of the rectum, but providing no evidence for either pelvic lymphadenopathy or distant metastasis.

immunohistochemical positivity for CD117 and CD34. Furthermore, there was also a mitotic activity of a 3 × 50 high power field (HPF), together with moderate cytological atypia and absence of necrosis.

TC confirmed the sonographic findings of the presence of a mass with a marked, irregular, eccentric thickening of the lateral left wall of the lower third of the rectum, but there was no evidence for either pelvic lymphadenopathy or distant metastasis (Figure 2).

The patient underwent nerve-sparing, anterior surgical resection of the rectum, with removal of the mesorectum, followed by colo-anal anastomosis and the formation of a protective colostomy. The post-operative period was perfectly normal, with no complications, and the patient was sent home on d 10. The colostomy was closed up two months after surgery.

Histological examination showed a submucous, intraparietal mass measuring 4 cm × 3 cm × 2 cm, with a central necrotic area and a completely healthy mucosa. The morphological aspect suggested a spindle-cell GIST, a diagnosis confirmed by the immunohistochemical investigation. Both CD117 and CD34 were, in fact, extremely positive (+++), and the mitotic index was < 5 × 10 HPF. The neoplasia was 5 cm away from the margin of the distal resection.

The patient did not undergo adjuvant therapy with imatinib. After 30 mo of follow-up, he showed no signs of either local regression or distant metastases.

DISCUSSION

GIST is an uncommon mesenchymal tumour located throughout the gastrointestinal tract and expresses CD117, a tyrosine-kinase growth factor receptor and the most important GIST marker[1]. CD117 also serves as the target for drug therapy with imatinib, a selective tyrosine-kinase receptor inhibitor that is at present the only promising chemotherapeutic drug for the treatment of patients with advanced GIST, although complete surgical resection remains the most effective treatment for such a tumour[6].

GISTs are most commonly found in the stomach (60%-70%), followed by the small intestine (20%-25%), only about 5% of all GISTs originate in the rectum[3,4].

The symptoms of GIST in the rectum do not generally differ from those of other rectal tumours and diagnostic work-up is also similar to that for any other rectal mass. Digital examination of the rectum, colonoscopy and transrectal ultrasound are essential for its diagnosis, together with preoperative biopsy, which plays a key role in the diagnosis of GIST, since it provides information on the immunohistochemical features and mitotic count. GIST typically expresses CD117, often CD34 and sometimes SMA and S-100, but its expressions vary depending on different sites. Miettinen *et al*[7] found that CD34 expression in rectal GIST is 92%, but only 50% in small intestinal GIST. Smooth muscle antigen (SMA) is most frequently seen in the small intestinal GISTs (47%), whereas it has been observed in only 14% of rectal GISTs. The reason for these variations has not yet been explained. Perhaps the differences in origin and their correlation with prognosis will be explained in the future.

Although endoscopy provides the diagnosis and biopsy material, a definite diagnosis is difficult if there is no mucosal invasion or extrinsic deformity. Most GISTs originate within the muscularis propria and most commonly have an exophytic growth pattern[8-10], seen on CT and MRI images. A focal, well-circumscribed mural mass is the most common finding and an infiltrated layer can be clearly assessed[11]. Furthermore, CT or MRI scanning is needed for the definition of local invasion level and for the detection of the presence of possible metastases.

Since the incidence of rectal GIST is much lower than that of GIST in the stomach or small intestine, the clinicopathological profiles of rectal GIST have not yet been accurately characterised, and there is therefore the tendency to validate the same prognostic factors for the latter as for such tumours at other sites, particularly gastric GIST. The most important and easily applicable histological criteria for prediction of GIST are its size and mitotic rate[12-15]. A rate of ⩽ 5 mitoses per 50 HPF is commonly used as a limit for a tumour with expected benign behaviour, and according to a large study, this can discriminate between benign and malignant tumours, especially gastric GISTs[12]. Tumours of 2 cm in diameter

are generally expected to behave in a benign fashion. Tumours of < 5 cm in diameter are associated with a better survival rate than those of 5 cm-10 cm in diameter, which in turn have a better prognosis than those of > 10 cm in diameter. Degrees of cellularity and atypia have also been suggested as useful criteria, but their reproducibility is more problematic. The epithelioid phenotype, which seems to lead to a worse outcome, together with symptoms lasting for at least a year, might be considered as further prognostic factors.

It is generally agreed that complete surgical resection with negative tumour margins is the principal curative procedure for primary and non-metastatic tumours, particularly for those at a low risk[8,16-18]. For rectal GIST, various surgical procedures may be considered, including local excision, anterior resection of the rectum and abdomino-perineal resection. The choice of procedure depends on tumour size and location[4,19]. Although not essential for the purpose of oncological radicality, we ourselves prefer to remove the mesorectum, which makes it much easier to spare the nerves of the lower hypogastric plexus, reduces intraoperative bleeding and guarantees more locoregional radicality, and this, in our opinion, also reduces the rate of local relapse. Neoadjuvant imatinib may enhance the resectability of inoperable malignant GIST and may allow for optimal surgical timing. Therapy with imatinib is also used in the adjuvant post-operative treatment of tumours at a high risk or in cases of incomplete surgical resection. It has now been more or less accepted that imatinib is a valid treatment for advanced or metastatic tumours, but further evidence for the efficiency of this drug is needed in the case of high risk tumours and for the neoadjuvant therapy.

In conclusion, rectal GIST should be included in differential diagnosis when a tumour in the rectum is detected although it is extremely rare. The diagnostic work-up of rectal GIST is exactly the same as that advised for any other type of rectal neoplasia. There is no doubt that biopsy of the tumour is an essential diagnostic instrument, since it can reach a certain preoperative diagnosis by means of the immunohistochemical characterisation of CD117 and CD34. Finally, the lack of a large series of patients under long-term follow-up observations makes it difficult to assess the necessary extent of surgical resection for rectal GIST and the indication for treatment with imatinib. Further studies are necessary to establish the most effective treatment strategy for patients with rectal GIST.

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CASE REPORT

Two cases of duodenal obstruction due to a congenital web

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Abstract

We present two cases of windsock deformity; both were rare in location and one had a rare associated anomaly. In the first case, the windsock was observed in the fourth part of duodenum, causing partial intestinal obstruction. In the second case, the windsock was located in the third part of the duodenum.

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Key words: Windsock deformity; Newborn; Duodenum; Obstruction

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INTRODUCTION

Windsock deformity (WD) is a rare anomaly. Fewer than 100 cases of WD have been reported[1-3]. The second part of the duodenum is the most common site of WD[1]. Associated anomalies, such as intestinal malrotation and a preduodenal portal vein, are well known. Presentation of a fenestrated duodenal web with acute pancreatitis has also been reported[4]. We report two cases of windsock deformity; both were rare in location and one had a rare associated anomaly.

CASE REPORT

Case 1

A 19 mo-old boy was admitted to the Clinic of Pediatric Surgery at the University of Yuzuncu Yil, Van, Turkey, with abdominal distention and vomiting. He had tolerated only milk and fluids since birth. The parents were children of first-degree relatives and had six living children. Two brothers of the case died immediately after birth and one sister died at an age of eight months, of unknown causes.

On physical examination, epigastric distention was present, but no mass or organomegaly was palpated. Abdominal X-ray on admission showed a dilated stomach (Figure 1). An upper gastrointestinal contrast study demonstrated an axial rotation appearance of the stomach. The transition time of small intestine content was evaluated as normal.

At laparotomy, the stomach was significantly dilated and the pylorus seemed to be completely erased at the junction. The wall thicknesses of the duodenum at the first and second parts were increased and similar to the thickness of the antral wall. The jejunoileal walls were thinner than normal and were empty at the distal end. Attempts were made to pass a nasogastric catheter to the jejunum, but the catheter could not go beyond the fourth part of the duodenum. Normal saline, given from the nasogastric catheter, could not pass freely to the distal end, and caused distention of the proximal duodenum. A longitudinal incision was made from the antimesenteric border at the transitional zone. A web, which caused an obstruction just before the Treitz ligament, was observed. In the centre of the web was a hole with a diameter of about 3-4 mm. The web was excised circumferentially. The vertical incision was closed. The nasogastric catheter was taken out on the seventh postoperative day. Abdominal X-rays at early and late postoperative days were normal. A contrast study in the fourth week after the operation revealed a normal duodenal appearance.

Case 2

A 5 mo-old girl presented to the same institution with abdominal distention and bilious vomiting, mostly seen after meals, for three months. At physical examination, she had mild epigastric distention. There was no mass or organomegaly in the abdomen on palpation. Hematologic parameters and biochemical laboratory findings were normal.

An upper gastrointestinal contrast study pre-operatively showed a dilated stomach. The duodenum was dilated in the first and second parts, but the third part was narrow (Figure 2). Abdominal ultrasonography revealed a normal liver size with a hyperechogenic lesion of 30 mm × 25 mm × 20 mm in size, compatible with hemangioma at the caudate lobe.

At laparotomy the duodenum was dilated and the wall

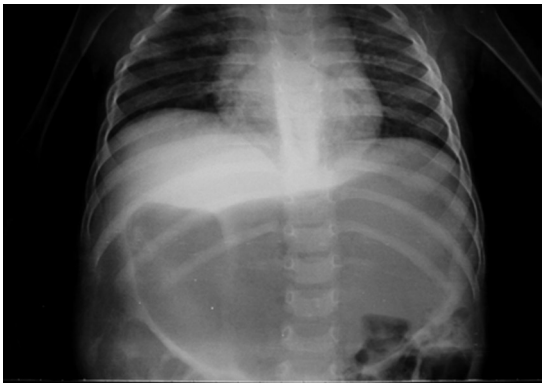


Figure 1 Pre-operative dilated stomach of the first case, which is distended with gas.

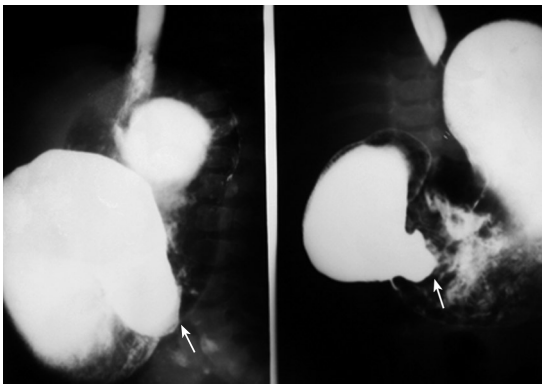


Figure 2 Pre-operative contrast study of case two showing a distended duodenum with contrast medium. Obstruction due to the web can be seen (arrow).

thickness was increased. Duodenotomy was performed longitudinally on the antimesenteric side after palpation of a membranous structure in the lumen. A web was seen and a windsock deformity was diagnosed. The opening in the middle of the web was catheterized with a 12 CH catheter (Figure 3). The web was excised circumferentially and the enterotomy was closed.

During exploration, a lesion localized in the caudate lobe of the liver 25 mm × 30 mm in size, elastic in nature and compatible with hemangioma was detected. Neither biopsy, nor excision was performed. The nasogastric catheter was pulled out postoperatively at sixth day. Oral feeding was tolerated well and she was discharged from the hospital on the 12th postoperative day.

DISCUSSION

Only 100 cases of WD have been reported in the literature[1-3]. The second part of the duodenum is the most common site, representing 85% to 90% of all WD cases. The third and fourth parts of the duodenum represent 20% and 10% of WD cases, respectively[1,5].

The WD in our first case was in the fourth part of the duodenum, which is a very rare location. The duodenum undergoes a solid phase; between the eighth and tenth week of gestation, the duodenal lumen is re-established by the gathering of vacuoles, and re-canalization occurs. Insults

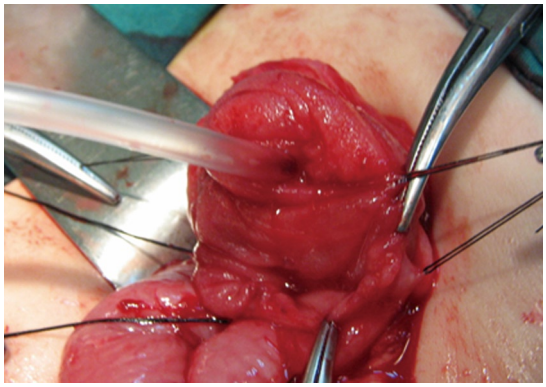


Figure 3 Duodenotomy revealing catheterized windsock deformity in the distal position. The web and its opening can be seen.

during this crucial period of development are believed to result in a failure of re-canalization and consequent atresia, stenosis and webs[5,6]. The resultant obstruction causes dilatation in the proximal duodenum and stomach, as well as hypertrophy and distention of pylorus[5].

Abdominal distention and vomiting were common complaints in each of our cases. Our first case was followed up by a pediatrician with diagnoses of constipation and growth failure. The great majority of individuals with WD are asymptomatic. A significant proportion of patients have a later onset of symptoms, and in some, the diagnosis is delayed until adulthood[7]. Clinical presentation may be characterized by nonspecific abdominal symptoms. Abdominal discomfort is usually located in the epigastria, right upper abdomen or umbilical area, and is made worse or brought on by eating and relieved by vomiting, belching or assuming a certain posture[1,5].

WD can be an incidental finding of upper gastrointestinal barium examination. In the second case, a pre-operative upper gastrointestinal study revealed the intraduodenal web. One of the most important features of duodenal diverticulum is the retention of barium in the sac. Barium retention for 6 or more hours is diagnostic[1,3].

Associated anomalies, such as intestinal malrotation and a preduodenal portal vein, are well recognized. Pancreatitis has also been reported in individuals with intraluminal duodenal diverticula[4,5]. Superior mesenteric artery syndrome is a very rare condition in childhood, most frequently resulting in duodenal obstruction in the adult population[12]. Bleeding from ulcers at the diverticulum[8], obstruction from gallstones[9] and marbles[3], as well as cholangitis[10] have been described. Hepatic hemangioma is an unusual finding in the pediatric population and is more common in older children and adolescents than in neonates and infants[1,11]. We discovered, incidentally, the hemangioma during US for the diagnosis of intestinal pathology.

Duodenotomy with web excision was performed in both of our cases. Two basic options for the repair of duodenal obstruction secondary to a web or atresia are presented: duodenoduodenostomy and duodenotomy with excision of the web. The web should be defined with the aid of a tube trough in the stomach, and the ampulla of Vater should be carefully identified prior to excision.

The advent of improved pediatric flexible fiberoptic endoscopes and fiberoptic laser technology makes endoscopic ablation of duodenal webs and windsocks in the newborn possible[5,9].

We aimed to present two cases of WD showing rare locations in the fourth and third part of the duodenum from the same institution.

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai - Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation - Management of Adeno- carcinomas
Email: robert.giuli@oeso.org

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008
June 4-7, Helsinki, Finland

The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008
June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
Email: meetings@imedex.com

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
Email: meetings@imedex.com

June 25-28, Lodz, Poland
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E-mail: office@epc-iap2008.org
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June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
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11th World Congress of the International Society for Diseases of the Esophagus
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III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses
September 24-27, Nantes, France
Third Annual Meeting
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16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Brisbane, Australia
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1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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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]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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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]
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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]
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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
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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/eid.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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